MEASUREMENT OF HUMAN GLUTATHIONE S-TRANSFERASES BY RADIOIMMUNOASSAY

BY

ALEXANDER FORBES HOWIE

A thesis submitted for the degree of

Doctor of Philosophy, University of Edinburgh

1990

DECLARATION OF ORIGINALITY

I declare that the work presented herein and
the composition of this thesis is my own.

Alexander Forbes Howie

ACKNOWLEDGEMENTS

Firstly, I wish to thank my supervisor, Dr. G.J.Beckett for his excellent advice, guidance, interest and patience throughout this project. I am also grateful to Dr J.D. Hayes, particularly for his advice on protein purification methods.

Many colleagues have helped me during this work and it is a pleasure to thank them. The staff in the Faculty Animal Area for their assistance in raising antibodies. Staff at Medical Illustrations, University of Edinburgh for the art work and photography. Professor D.T. Baird and Dr M.McIntyre for providing some of the tissues used in this study. Drs. L.M.Aldridge, D.Bell, J.G.Douglas, R.J.Fergusson, P.C.Hayes, A.W.Patrick, P.W.H.Rae, D.C.Ray and C.G.Wathen, all of whom collected blood, bile or lavage samples from patients, without which this project would not have been possible. The staff in the Department of Clinical Chemistry, University of Edinburgh, many of whom willingly provided their own blood for my studies. Dr W.R.Miller and his colleagues from the Department of Surgery, University of Edinburgh, for providing the breast cancer cytosols and analysing their oestrogen receptor status. Dr C.R. Wolf and his colleagues (particularly M.J.Glancey) from the Imperial Cancer Research Laboratory, Hugh Robson Building, University of Edinburgh for providing the matched tumour cytosols studied in Section 6 and performing the Western blot analysis described in that section. Dr R.C. Strange and his colleagues from The Department of Postgraduate Medicine, Keele University and Dr R.Hume, Special Care Baby Unit, Royal Infirmary, Edinburgh who provide the human cytosols studied in Section 9.

I gratefully acknowledge Professor L.G.Whitby for allowing me to undertake this project and for the use of his laboratory facilities. Dr A.F.Smith has also to be thanked for his support, and assistance with statistical and word processing procedures.

I thank my wife and parents for their support and encouragement.

Finally, a special thank you goes to Miss E.T.Campbell (Aunt Betty) for her encouragement, faith and support throughout this project.

ABSTRACT

A purification scheme was devised for human glutathione S-transferase (GST) pi that resulted in a high yield of homogeneous protein from human placenta, lung and erythrocytes. Polyclonal antisera against GST pi from lung and placenta were raised in eight rabbits. The GST pi proteins purified from placenta, lung and erythrocytes were found to have identical isoelectric points (pI=4.8) and subunit molecular weights (Mr=24 800), and were found to be immunologically identical. The antibody demonstrating the best specificity and sensitivity was employed to develop a radioimmunoassay (RIA) for the measurement of GST pi.

Using the RIA, GST pi concentrations were measured in the plasma of patients diagnosed as having cancer of the bronchus. Concentrations of the enzyme were significantly higher ($P<0.01$) than those measured in a control group of patients with respiratory disorders other than malignancy. To prevent spuriously high results for GST pi concentrations caused by platelet release, the blood for analysis was collected into 'Thrombotect' tubes, which contain inhibitors of platelet activation.

The RIA developed for GST pi and also existing RIA for GST B_1, GST B_2 and GST μ were used to determine GST levels in a variety of biological fluids and tissues. Measurement of the individual GST isoenzyme expression in normal and tumour cytosol prepared from human lung, colon and stomach showed that the concentration of GST pi was significantly increased in tumour tissue relative to paired normal tissue. The expression of GST B_1 and GST B_2, the predominant isoenzyme in normal liver, kidney and stomach, decreased dramatically in tumour tissue from these organs. In breast cancer cytosols significant differences in GST expression were also observed between oestrogen receptor-rich and oestrogen receptor-poor tumours. The proportion of individuals with cancer found to express the polymorphic GST isoenzyme GST μ was not significantly different from the expression rate of the isoenzyme in control populations. The expression of the various GST isoenzymes in development showed that there was a decrease in GST pi expression in liver and lung as the fetus matured, while the alpha class GST showed an increase in expression throughout development.

Purification of GST isoenzymes from bronchoalveolar lavage and gall bladder bile by affinity chromatography showed that GST pi constituted the major isoenzyme in bile,

and it is postulated that GST pi acts as a carrier protein of toxic, non-substrate, ligands to remove as yet unidentified substances from biliary epithelial cells and prevent their reabsorption. Concentrations of GST B_1 and GST B_2 were found to be significantly raised ($P<0.02$) in bronchoalveolar lavage fluid obtained from the suspected abnormal area of lung compared with the presumed normal area of lung, in patients later diagnosed as having cancer of the bronchus, whilst in patients with non-malignant respiratory disorders no significant difference in GST concentration was found.

Plasma GST B_1 measurements were used to investigate hepatocellular integrity in adults following hypoglycaemia and in children exposed to isoflurane or halothane anaesthesia. Hypoglycaemia produced a significant increase in plasma GST B_1. Abnormalities in plasma GST B_1 were greater in children receiving halothane compared to the children who received isoflurane.

These studies indicate that GST pi measurements by RIA may provide a useful tumour marker for cancer of the bronchus, whilst GST B_1 measurements in bronchoalveolar lavage may be useful in the diagnosis of suspected lung malignancy. The studies on plasma GST B_1 measurements confirm previous studies that suggested such measurements provide an extremely sensitive index of hepatocellular integrity.

CONTENTS

CONTENTS

CONTENTS

CONTENTS

CONTENTS

CONTENTS

ABBREVIATIONS

AAFB	acid and alcohol fast bacteria
ALT	alanine aminotransferase
ALP	alkaline phosphatase
ATP	adenosine triphosphate
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CDNB	1-chloro-2,4-dinitrobenzene
cpm	counts per minute
CuOOH	Cumene hydroperoxide
CV	coefficient of variation
DARS	donkey anti-rabbit IgG serum
DNA	deoxyribonucleiic acid
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme-linked immunosorbant assay
ENT	ear, nose and throat
ER	oestrogen receptor
ERCP	endoscopic retrograde cholangiopancreatography
FAEE	fatty acid ethyl ester
FPLC	fast protein liquid chromatography
GCMS	gas-chromatographic mass spectrometer
GSH	reduced glutathione
GSSH	oxidised glutathione
GST	glutathione S-transferase
γGT	γ-glutamyl transferase
HbA_1	haemoglobin A_1
HRP	horseradish peroxidase
IEF	isoelectric focusing
IPPV	intermittent positive pressure ventilation
IV	intravenous
Mr	molecular weight
mRNA	messenger ribonucleic acid
NADPH	dihydronicotinamide adenine dinucleotide phosphate
NRS	normal rabbit serum

NSB	non-specific binding
PF_4	platelet factor four
RIA	radioimmunoassay
RNA	ribonucleic acid
SD	standard deviation
SDS/PAGE	sodium dodecyl sulphate/polyacrylamide-gel electrophoresis
SE	standard error
sGPX	selenium-dependent glutathione peroxidase
SMAC	sequential multiple analyser with computer system
SR	spontaneous respiration
TCA	trichloroacetic acid
TEMED	NNN'N-tetramethyl ethylenediamine
TFA	trifluoroacetic acid
TFA-RSA	trifluoroacetic acid rabbit serum albumin conjugate
tGPX	total glutathione peroxidase
TRIS	2-amino-2-(hydroxymethyl)propane-1,3-diol

Section 1 : INTRODUCTION

The multiple isoenzymes of the glutathione S-transferases (GST; EC 2.5.1.18) catalyse the conjugation of reduced glutathione (GSH) with numerous electrophilic, hydrophobic compounds in the first step of mercapturic acid formation (Boyland and Chasseaud, 1969). These isoenzymes are often present in high cellular concentrations, thus the relatively low substrate specificities and catalytic efficiencies reported for GST are counterbalanced by the abundance and versatility of these enzymes. Such qualities appear well suited to a family of proteins whose function is to protect cells against the toxic effects of the multitude of exogenous or endogenous compounds that may be encountered. This family of GST enzymes may also be involved in the biosynthesis of leukotrienes and prostaglandins (Mannervik *et al.*, 1984; Christ-Hazelhof *et al.*, 1976).

Certain GST isoenzymes also exhibit peroxidase activity and are able to reduce diverse organic hydroperoxides to their respective alcohols. Potential substrates including fatty acids and DNA which have been subject to peroxidation, hence the peroxidase activity of GST may represent part of a repair mechanism for both DNA and lipids (Ketterer *et al.*, 1987).

In addition to their catalytic functions, the GST also exhibit ligand binding properties for a variety of organic compounds such as bilirubin, steroids and bile salts. A property reflected in a previous term for certain GST isoenzymes 'ligandin'. Thus GST may serve as intracellular storage sites or transport proteins for hydrophobic compounds, including both xenobiotics and endogenous metabolites.

In the last decade numerous publications have been concerned with the properties of the GST enzymes and the factors which are involved in their regulation and expression. As each GST isoenzyme provides a protection against a different spectrum of toxic chemicals, particular emphasis has surrounded the possible role of GST enzymes in drug resistance, whether it be intrinsic or acquired (Review: Hayes and Wolf, 1988). Several models have been studied, and it has been shown that increased resistance to cytotoxic insult can be accompanied by over-expression of GST enzymes.

Another related area where GST involvement has been under study is in its possible role in carcinogenesis. With elevated levels of the GST pi isoenzyme being reported in tumour tissues and preneoplastic lesions (Sato *et al.*, 1984). Thus the plasma measurement of GST pi isoenzyme, may have a role as a tumour marker for diagnosing or monitoring the progression of certain cancers. The plasma measurements of the GST isoenzymes GST B_1 and GST B_2 have already been shown

to be sensitive markers of hepatic integrity (Beckett and Hayes, 1987).

1.01 : Glutathione: the substrate

Glutathione is a tripeptide with the sequence, γ-glutamic acid-cysteine-glycine and may exist in the reduced (GSH) or oxidised (GSSG) form (figure 1a). The γ-glutamyl linkage between the N-terminal glutamate and the cysteine is unusual in peptides and it is thought that this may provide a mechanism of resistance to degradation by cellular proteases. GSH has a net negative charge at physiological pH and is highly water soluble with the nucleophilic thiol group able to interact with electrophilic compounds.

Glutathione is ubiquitous in eukaryotic cells being implicated in a number of cellular functions. It is the most prevalent cellular thiol and the most abundant low molecular weight peptide present in cells. Glutathione acts as a reducing agent and an antioxidant, serves as reservoir for cysteine, participates in detoxification reactions for xenobiotics and metabolism of numerous cellular compounds, is required for the synthesis of some prostaglandins and leukotrienes, and may be involved in cell cycle regulation (Meister and Anderson, 1983; Deneke and Fanburg, 1989).

In man the reduced form of glutathione is present in cells in concentrations ranging from 0.5 to 10 mmol/L depending on the organ, compared to the much lower concentrations of the oxidised form (4 to 10 μmol/L). Hepatocellular concentrations of GSH are maintained by achieving a balance between the rate of synthesis and the rate of utilization. GSH may be formed either from reduction of the oxidised form by glutathione reductase or by the direct synthesis from constituent amino acids (Kaplowitz *et al.*, 1985).

Many enzymes utilize GSH as a substrate including glutathione peroxidase, glutathione reductase, glyoxalase, glutathione transhydrogenase and glutathione S-transferase.

Conjugation of electrophilic xenobiotics with the sulphur atom of GSH by GST usually results in a decrease in the reactivity and toxicity and an increase in the solubility of the compound. The conjugation of xenobiotics or endogenous metabolites with GSH is the first step in the formation of mercapturic acids, which may be readily excreted into urine (Boyland and Chasseaud, 1969). The anionic nature of GSH and its low molecular weight favours the excretion of the GSH conjugate in bile (Chasseaud, 1974).

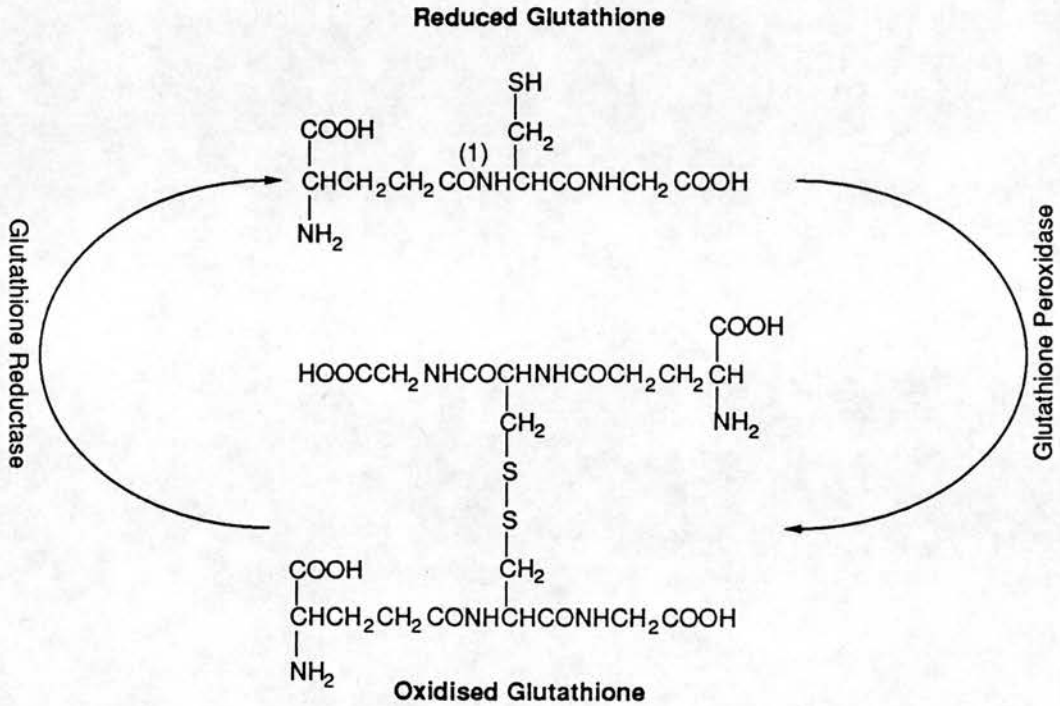

Figure 1a: The inter-relationship of reduced glutathione with oxidised glutathione (glutathione disulphide; GSSG) and their chemical structures.
[(1), γ-glutamyl peptide link]

1.02 : The Mercapturic acid pathway

The mechanism of mercapturic acid biosynthesis and the involvement of GSH conjugates was established in 1959 (Barnes *et al.*, 1959; Bray *et al.*, 1959a,b) and a comprehensive review of the role of GSH and GST in mercapturic acid biosynthesis was described by Boyland and Chasseaud (1969). Briefly the thioesters, formed by the reaction catalysed by GST, are converted to mercapturic acids in three separate stages (figure 1b):

1) The removal of the γ-glutamyl moiety by the action of γ-glutamyl-transferase.
2) The removal of glycine by the action of a dipeptidase.
3) The N-acetyl-CoA linked acetylation of the cysteine conjugate to form a N-acetylcysteine thioether, which is the mercapturic acid.

Hepatic GSH conjugates and catabolites are excreted in bile. There may be further catabolism in the intestine, and conjugates may undergo enterohepatic circulation and/or be excreted in the faeces (Chasseaud, 1976). Because of their high solubility, mercapturic acids and related conjugates are eliminated from tissues and blood very rapidly.

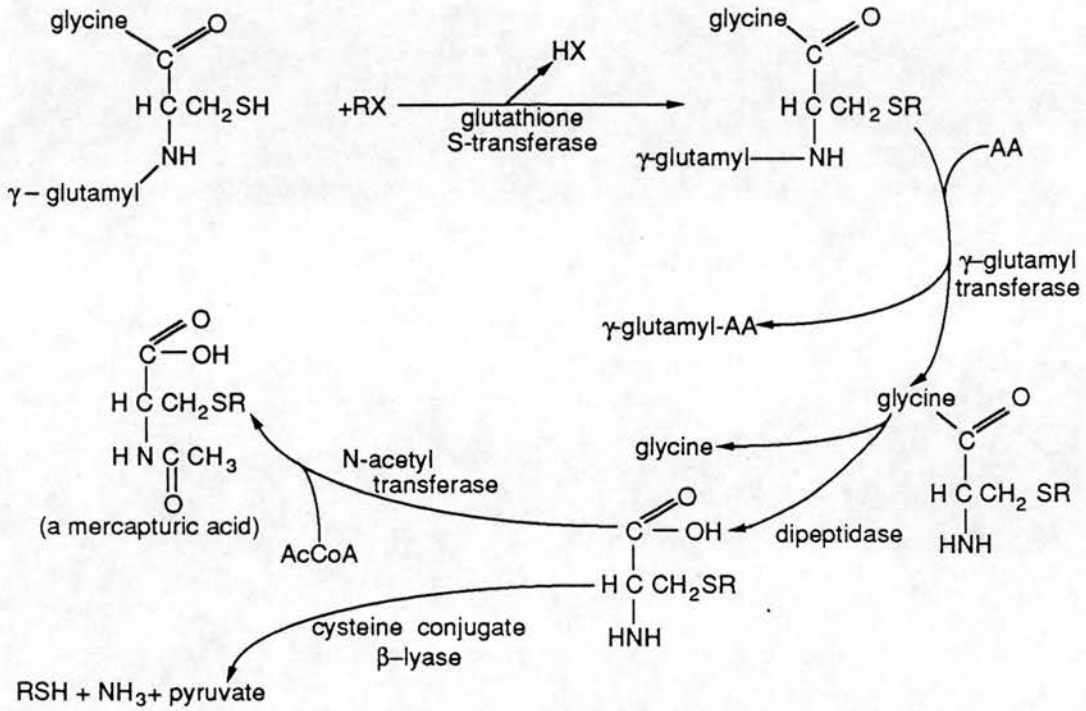

Figure 1b: Conjugation of an electrophilic compound (RX) with glutathione is catalysed by glutathione S-transferase (the conjugate may be excreted directly into bile). Catabolism of non-excreted or reabsorbed glutathione conjugates usually takes place in the kidney where γ-glutamyl transferase catalyses the transfer of the γ-glutamyl moiety to neutral amino acids (AA) or peptides. After which, cysteinyl-glycinase (dipeptidase) catalyses the hydrolysis of the cysteinyl-glycine peptide bond, with the release of glycine. The cysteinyl derivative can then be converted by N-acetyl transferase to a mercapturic acid by N-acetylation with acetyl CoA (AcCoA). Alternatively, the cysteine conjugate can be converted to the thiol derivative, pyruvate and NH_3 by cysteine conjugate β-lyase.

1.03 : Historical overview of the GST

The publication of the paper 'The reaction of 1,2-dihydronaphthalene and 1,2-epoxy-1,2,3,4-tetrahydronaphthalene', by Booth *et al.* (1960), was of particular importance as it was the first paper to report enzymic catalysis of GSH conjugation by the rat-liver soluble supernatant fraction (cytosol). The following year Booth *et al.* (1961) demonstrated GST activity in rat liver cytosol with a number of electrophilic substrates, including 1,2-dichloro-4-nitrobenzene, benzyl chloride, bromoethane and bromsulphthalein. In the same year Coombes and Stakelum (1961), also showed that rat-liver cytosol catalysed the GSH conjugation of bromsulphthalein.

Shortly after these initial reports, it was recognised that several types of GST activity occurred in rat liver. The enzyme(s) that catalysed the conjugation of GSH with aromatic halogen and nitro compounds, was different from the GST which catalysed the conjugation of GSH with aliphatic halogen compounds (Johnson, 1963) and the various forms of GST that were described, were named according to the type of reaction that they catalysed (Boyland and Chasseaud, 1969; Chasseaud, 1974). Hence, glutathione S-aryltransferase conjugated GSH to aromatic and other cyclic compounds containing labile halogens or nitro groups, glutathione S-alkyltransferases utilized alkylhalides as substrates, glutathione S-aralkyltransferases were specific for aralkylhalides and esters and, glutathione S-epoxide transferase conjugated various epoxides with GSH. At the time when this nomenclature was employed homogeneous enzyme preparations were not available but as various GST were purified it became evident that the separate dimeric isoenzymes had overlapping substrate specificities and the nomenclature based on the structure of the substrate was abandoned.

The development of affinity chromatography matrices such as glutathione-Sepharose (Simons and Vander Jagt, 1977) and S-hexylglutathione-Sepharose (Guthenberg *et al.*, 1979) greatly facilitated the purification of GST isoenzymes and in conjunction with chromatofocusing has meant that many new GST forms have been identified and characterized in species as diverse as plants (Mozer *et al.*, 1983), bacteria (Di Ilio *et al.*, 1988a), yeast (Tamaki *et al,* 1989), insects (Jansen *et al.*, 1982), fish (Nimmo, 1987) as well as, numerous mammals including man.

At the same time as the early experiments on GST isoenzymes were being reported, reports were accumulating concerning a cytosolic protein from rat liver which had a high affinity for various endogenous and exogenous organic anions. Ketterer *et al.* (1967) isolated a carcinogen-binding protein named 'basic azo-dye carcinogen-binding protein (β-ABP)'. Morey and Litwack (1969) isolated a cortisol metabolite

binding protein termed 'Corticosteroid Binder I' and Levi *et al.* (1969) isolated a bilirubin- and bromsulphthalein-binding protein called 'Y protein'. After collaboration between the three laboratories it was found that these proteins were identical and the protein was renamed 'Ligandin' (Litwack *et al.*, 1971). As ligandin was shown to be capable of binding a large number of organic anions, it was proposed that ligandin functioned as a carrier protein transferring hydrophobic and anionic compounds such as bilirubin and bile salts across the hepatocyte for excretion into the bile (Levi *et al.*, 1969; Reyes *et al.*, 1971; Fleischner *et al.*, 1972). Ligandin was also shown to bind covalently certain carcinogens, notably azo-dye carcinogens and metabolites of polycyclic hydrocarbons (Ketterer *et al.*, 1967; Ketterer, 1972).

Although Kaplowitz *et al.* (1973) demonstrated an association between bromsulphthalein binding to ligandin and its conjugation with glutathione, the discovery that ligandin was a GST is accredited to Jakoby and colleagues in collaboration with Arias and his group who reported that ligandin was identical to glutathione S-transferase B (Habig *et al.*, 1974). The relationship between ligandin and GST in the literature, has been complicated by the fact that many of the early preparations of the protein were heterogeneous GST mixtures. Hayes *et al.*, (1979) demonstrated that ligandin was in fact a homodimer comprising Ya subunits (nomenclature introduced by Bass *et al.*, (1977) see Table 1a.) whereas, GST B was found to be heterodimer comprising Ya and Yc subunits. The term 'Ligandin' is now seldom employed to describe this cytosolic binding protein and care is required when interpreting historical data, since different laboratories may not be describing the same protein.

1.04 : Nomenclature of the GST

As mentioned in the previous section the first classification system of cytosolic GST based on differences in substrate utilisation (Boyland and Chasseaud, 1969) was soon found to be inappropriate and replaced. The purification scheme devised by Habig *et al.* (1976), for the isolation of distinct GST isoenzymes from rat liver, described several isoenzymes named AA, A, B, C, D and E but even this scheme failed to resolve all the GST forms which occur in rat liver not to mention extra-hepatic forms. Thus the GST nomenclature based on this method was incomplete and later superseded.

The cytosolic GST isoenzymes are dimeric and it was their quaternary structure that provided a basis for the rat nomenclature used currently, the various GST being named after the subunits from which they comprise. This system of nomenclature was

introduced by Bass *et al.* (1977), who named the major subunits of the soluble bromsulphthalein-binding Y fraction of rat liver as Ya, Yb and Yc, according to their mobility on SDS/PAGE. The subunit mobility system has been adopted widely and has been modified to include Yb_1 and Yb_2, Yf, Yk and Yn_1 and Yn_2 (Hayes, 1984, 1986; Hayes and Mantle, 1986a; Ishikawa *et al.*, 1987) as these additional subunits were identified. The relative mobilities of the subunits on SDS/PAGE in 12.5% polyacrylamide resolving gels are Yf>Yk>Ya>Yn>Yb>Yc (Hayes, 1986; Hayes and Mantle, 1986a). An alternative nomenclature, where subunits were assigned Arabic numerals, was proposed by Jakoby *et al.* (1984). The relationship between both forms of current rat nomenclatures is shown in table 1a which also includes some trivial GST designations.

The human GST isoenzymes first purified were denoted by Greek letters and allocated to three classes, based on isoelectric points, and termed basic, neutral and acidic. The first isoenzymes to be identified were named as follows: five enzymes with basic isoelectric points called GST α, β, γ, δ and ϵ (Kamisaka *et al.*, 1975); two neutral GST termed GST μ (Warholm *et al.*, 1981) and GST ψ; and three acidic GST from erythrocytes, placenta and lung termed ρ (Marcus *et al.*, 1978), π (Guthenberg and Mannervik, 1981) and λ (Hayes *et al.*, 1986) respectively. As more human GST isoenzymes were described, it was found that they did not fit readily into the basic, neutral or acidic classes, and Mannervik *et al.* (1985) introduced the alpha, mu and pi-classification scheme, based on structural, immunological and enzymatic properties, to describe mammalian (species-independent) GST isoenzymes that shared similar qualities. In man the GST isoenzymes have the following homodimeric structure: alpha-class GST, Ya-type subunits; mu-class GST, Yb-type subunits; and pi-class GST, Yf-type subunits.

The human alpha-class GST were originally described by Kamisaka *et al.* (1975), who purified five basic GST forms (α, β, γ, δ and ϵ) from liver and postulated that these enzymes were encoded for by a single gene and the multiple forms were generated by a postsynthetic modification, possibly deamination. Stockman *et al.* (1985,1987) also using human liver, purified and characterized three basic GST termed GST B_1B_1, GST B_1B_2 and GST B_2B_2, the subunits B_1 and B_2 being products of separate genes. This proposal has been validated by subsequent amino acid sequence data (Hayes *et al.*, 1989) and DNA sequence information (Tu and Qian,

1986; Rhoads *et al.*, 1987). The B_1B_1, B_2B_2 and B_1B_2 correspond to GST ϵ, γ and δ. In the original papers of Beckett and Hayes where the development of radioimmumoassays for GST B_1 and GST B_2 subunits are described (Hayes *et al.*, 1983; Beckett and Hayes, 1984), the GST B_1 and GST B_2 subunits were called basic and N/A2b respectively; the term N/A standing for neutral/acidic. The confusion over the GST B_2 nomenclature arose because it was retained by the diethylaminoethyl (DEAE-) anion exchanger and was therefore wrongly thought to have a neutral/acidic isoelectric point. The terms GST B_1 and GST B_2 are used throughout this thesis when referring to specific subunits, whereas alpha GST isoenzymes refers to the class of isoenzymes composed of YaYa type subunits.

Alpha-class GST isoenzymes have been reported to be present in a number of extra-hepatic organs such as kidney (Tateoka *et al.*, 1987), testis (Aceto *et al.*, 1989) and prostate (Tew *et al.*, 1987) these isoenzymes probably corresponding to those identified in liver (comprising YaYa type subunits). One exception to this was a report of the identification of a novel GST (isoelectric point = 9.9) in human skin homologous with class alpha GST YcYc in the rat (Del Boccio *et al.*, 1987).

The human pi-class GST has been identified in all tissues so far examined. This pi-class GST has been referred to as "GST-π and "GST-P" when isolated from placenta, "GST-λ" when isolated from lung, and "GST-ρ" when isolated from erythrocytes. The general name acidic GST (because of its low isoelectric point, pI=4.8) has also been used. Only one gene for GST pi has been demonstrated (Cowell *et al. 1988*), also the studies undertaken here (section 3) have shown that GST pi purified from placenta, lung or erythrocytes is indistinguishable immunologically. Throughout this thesis, I use the term GST pi to refer to this acidic GST isoenzyme, comprising YfYf type subunits, irrespective of its tissue of origin.

The majority of the literature concerning the mu-class GST (comprising YbYb type subunits) has concerned the GST enzyme GST μ and its allelic variant GST ψ However, other enzymes have been described and these are discussed more fully in Section 7.

A completely separate Zymogram-based nomenclature scheme for human cytosolic GST isoenzymes has also developed based on a report by Board (1981), who, using starch-gel electrophoresis of cell extracts coupled with a GST activity stain, reported the existence of 3 separate GST loci termed GST1, GST2 and GST3. Further loci have been identified termed GST4, GST5 (Laisney *et al.*, 1984) and GST6 (Suzuki

et al., 1987), this nomenclature is discussed further in Section 7 and its relationship with individual subunit composition and alpha, mu and pi-class illustrated in table 1b.

In addition to the cytosolic GST enzymes, a human microsomal GST has been described (McLellan *et al.*, 1989), which is distinct in that it is a trimeric protein with three identical subunits of molecular weight 17300.

Table 1a: Alternative nomenclatures for the cytosolic GST subunits in the rat. The 'Y' designation is the system based on Bass *et al.* (1977). The numerical designation is that proposed by Jakoby *et al.* (1984) and the family designation is based on the proposal of Mannervik *et al.* (1985).

'Y' designation	Numerical designation	Trivial name	Family
YaYa	1-1	Ligandin**	alpha
YaYc	1-2	B**	alpha
YcYc	2-2	AA*	alpha
YkYk	8-8	K**	alpha
YlYl	10-10		alpha
YoYo	11-11		mu
Yb_1Yb_1	3-3	A*	mu
Yb_1Yb_2	3-4	C*	mu
Yb_2Yb_2	4-4	D*	mu
YnYn	6-6	N**	mu
Yb_1Yn	3-6	P**	mu
Yb_2Yn	4-6	S**	mu
ND	5-5	E*	mu
YfYf	7-7	P(YpYp)***	pi

ND represents no designation.
* represents nomenclature employed by Fjellstedt *et al.*, 1973; Habig *et al.*, 1974, 1976.
** represents nomenclature employed by Hayes *et al.*, 1979; Hayes, 1984, 1986.
*** Kitahara *et al.*, 1984.

Table 1b: Nomenclatures for human GST isoenzymes. ND represent not determine. For references 1-11 see next page.

GST Isoenzyme	Family	Subunit mol.wt. (by SDS/PAGE)	Isoelectric point	Zymogram-based nomenclature Locus[1-3]	Band-type
B_1B_1[4] (ϵ)[5]	alpha	25900	8.9	GST-2	1
B_1B_2[4] (δ)[5]	alpha	25900	8.75	GST-2	2-1
B_2B_2[4] (γ)[5]	alpha	25900	8.4	GST-2	2
Skin '9.9'[6]	alpha	27500	9.9	ND	
μ[7]	mu	26700	6.1	GST-1	2
ψ[8]	mu	26600	5.5	GST-1	1
ND	mu	ND	ND	GST-1	2-1
ϕ[9]	mu	26700	4.6	ND	
ND	mu	27300	5.2	GST-4	
ND	mu	27500	5.9	GST-5	
ND		26950	4.25	GST-6	light
ND	mu	27500	4.25	GST-6	heavy
π[10]	pi	24800	4.8	GST-3	
Microsomal[11]	ND	17300	ND		

References for Table 1b;

1. Board, (1981) Am.J.Hum.Genet. 33,36-43.
2. Strange *et al.*, (1984) Ann.Hum.Genet. 48,11-20.
3. Suzuki *et al.*, (1987) Ann.Hum.Genet. 51,95-106.
4. Stockman *et al.*, (1985) Biochem.J. 227,457-465.
5. Kamisaka *et al.*, (1975) Eur.J.Biochem. 60,153-161.
6. Del Boccio *et al.*, (1987) Biochem.J. 244,21-25.
7. Warholm *et al.*, (1983) Biochemistry 22,3610-3617.
8. Hayes, (1989) Clin.Chem.Enzym.Comms. 1,245-264.
9. Stockman & Hayes, (1987) Glutathione S-transferases and Carcinogenesis (Mantle, Pickett & Hayes, eds.) Taylor & Francis, pp.41-42.
10. Guthenberg *et al.*, (1979) Acta Chem.Scand. B33,595-596.
11. McLellan *et al.*, (1989) Biochem.J. 258,87-93.

1.05 : The structure and function of GST

The quaternary structure of cytosolic GST is based on the combination of two subunits from the same class. To date, GST with a dimeric combination of subunits from separate classes (alpha, mu or pi) have not been isolated nor has *in vitro* hybridization, after denaturation, been demonstrable (Boyer *et al.*, 1983). Why the cytosolic GST exist as dimers is not known, since it has been shown that individual subunits function independently and, from a catalytic viewpoint, there appears to be no reason why the GST should comprise more than one polypeptide (Mannervik, 1985).

Conjugation of electrophilic compounds with GSH involves interaction of the separate substrates with the GST enzyme, at distinct binding sites. Jakobson *et al.* (1977) proposed that each subunit contains an active centre composed of two binding sites, one with a high affinity for GSH, the other a hydrophobic pocket specific for the second electrophilic substrate. The catalytic mechanism by which GST afford an increased rate of conjugation with electrophiles is considered to be simple proximity effect, as the rate of enhancement of the reactions is not great, and the majority of the reactions may proceed non-enzymatically (Jakoby, 1978).

1.06 : Types of reaction catalysed by GST

The reactions catalysed by the GST are generally nucleophilic attack of GSH on various electrophiles. Electrophilic carbon in various forms is susceptible to GSH conjugation by one or more of the GST isoenzymes. For example, the GST catalyse GSH conjugation with epoxides, alkyl and aryl halides, and ring opening of lactones. Addition of GSH to activated alkenes and related compounds are also common. Displacements of molecules at elements other than carbon are also known, for example organic nitrates where the GSH attacks the electrophilic nitrogen and organic thiocyanates where the GST catalyse the nucleophilic attack by GSH on the sulphur atom.

The alpha-class GST have also been shown to exhibit peroxidase activity against organic peroxides. This activity of GST is referred to as selenium-independent glutathione peroxidase activity or Type II glutathione peroxidase activity, to distinguish it from the activity catalysed by the seleno-enzyme, glutathione peroxidase (Type I).

Other reactions also implicated to involve GST include steroid isomerisation, where GSH is not consumed but acts as a co-enzyme, the formation of prostaglandin D from prostaglandin endoperoxides and the biosynthesis of leukotriene C_4 monomethyl ester from leukotriene A_4 methyl ester. For a review of the majority of

these reactions see Chasseaud (1979).

Table 1c: Examples of a variety of biologically important substrates, utilised by the GST isoenzymes.

TYPE OF SUBSTANCE	SUBSTRATE
Arachidonic acid derivatives	leukotriene A_4 methyl ester Prostaglandin H_2
Carcinogens	aflatoxin B_1-8,9-oxide benzo(α)pyrene 7,8-diol-9,10 oxide
Anticancer agents	nitrogen mustards nitrosoureas anthroquinone
Products of membrane and DNA oxidation	fatty acid hydroperoxides 4-hydroxy alkenals DNA hydroperoxides

1.07 : Model substrates and inhibitors for GST subunit identification

Since many of the conjugation reactions of electrophiles with GSH may be followed spectrophotometrically, the catalysis effected by GST can be estimated simply. Individual cytosolic GST isoenzymes have been shown to display a distinctive spectrum of catalytic activities with certain substrates and substrates which can differentiate individual classes of GST have been described. These specificities are listed in table 1c, with the alpha-class demonstrating a high specific activity for Cumene hydroperoxide and Δ^5-Androstene-3,17-dione, the mu-class having a high specific activity towards Bromsulphthalein and *trans*-4-Phenyl-3-butene-2-one and the pi-class showing high a specific activity for the diuretic Ethacrynic acid.

The specific activities for the 'model' substrate for GST, 1-chloro-2,4-dinitrobenzene are also included, to highlight that each class of human GST exhibit markedly different specific activities even towards this 'model' substrate.

In addition to the individual subunits having distinctive catalytic properties, with regard to substrate specificities, they also differ with respect to inhibition of activity by various compounds. The use of specific inhibitors augments the use of various substrates to discriminate between GST subunits. The most frequently used inhibitors include Cibacron blue, Triphenyltin chloride, Bromsulphthalein, Haematin, Bilirubin and various Bile acids (Tahir and Mannervik, 1986).

Table 1c: Specific activities of human GST enzymes. Taken from Stockman et al. (1987) and Warholm *et al.* (1983). ND represents not determined.

		specific activity (μmol/min/mg)		
Substrate	**GST**	**alpha**	**mu**	**pi**
Ethacrynic acid		0.16	0.22	1.22
Cumene hydroperoxide		92	ND	0.11
Δ^5-Androstene-3,17-dione		8.0	0.12	0.01
Bromsulphthalein		<0.002	0.01	<0.01
trans-4-Phenyl-3-butene-2-one		0.0	0.45	0.02
1-Chloro-2,4-dinitrobenzene		117	272	212

1.08 : GST as transport proteins

The suggestion that hepatic anion transport involves the GST frequently appears in the literature and the mechanism of non-covalent binding of substances such as haematin, bromsulphthalein, bile salts and bilirubin, has been investigate by a number of research groups using a variety of methods including equilibrium dialysis and chromatography, quenching of intrinsic tryptophan fluorescence, circular dichroism and inhibition kinetics (Strange *et al.*, 1976,1977; Hayes and Mantle, 1986b; Listowsky *et al.*, 1988). Despite numerous studies controversy still persists regarding the nature and number of binding sites for each isoenzyme subunit.

The GST (primarily ligandin, alpha-class) have been implicated in bilirubin uptake, not only by binding studies but also from physiological observations. Levi *et al.*, (1969,1970) suggested that the unconjugated hyperbilirubinaemia, frequently found in neonatal humans and monkeys, results from a hepatic deficiency of Y protein (Ligandin), because in monkeys the amount of Y protein was significantly lower during fetal and early neonatal life than at 5 days after birth. The increase in the concentration of Y protein at 5 days coincided with the development of efficient clearance of bromsulphthalein from plasma. It has also been suggested that GST may have a role in the intrahepatic transport of conjugated bilirubin. In a case report of a 17 year-old male patient with Rotor's syndrome (mild, fluctuant conjugated-hyperbilirubinaemia) (Adachi and Yamamoto, 1987) it was found that the patients hepatic GST activity towards 1-chloro-2,4-dinitrobenzene reduced to about 0.2% of the mean activity of patients with other diseases.

Although the binding of bile salts to GST has received considerable attention, with the possible exception of lithocholic acid, it appears unlikely that GST act as carrier proteins for bile acids across the hepatocyte (Strange, 1984). Simple diffusion is a more likely mechanism of bile acid transport. The theory that GST may have a role in the intracellular transport of haem (a hydrophobic molecule produced in the inner mitochondrial membrane) has also been refuted recently (Kirschner-Zilber *et al.*, 1989), these workers using cultured cells, finding no association between haem synthesis and GST levels.

Listowsky *et al.*, (1988) investigated the binding of thyroid and steroid hormones, polycyclic aromatic hydrocarbons and related compounds to GST, and reported binding of broad specificity but low affinity was evident in many cases. This report suggested that GST, due to its high intracellular concentration, acted as a kind of intracellular safety net for many of these hydrophobic compounds, since if the levels

of the ligand exceed the capacity of the specific receptor or if there was no specific receptor (in the case of xenobiotics) cytotoxic effects would result unless binding to GST occurred.

1.09 : Covalent binding of carcinogens by GST

The GST are reported to reduce the susceptibility of the liver to carcinogenesis, not only through their ability to detoxify carcinogens but also by their ability to bind covalently highly electrophilic compounds. Azo-dye carcinogen metabolites and activated metabolites of 3-methylcholanthrene have been shown to combine with rat ligandin (Ketterer *et al.*, 1976). The Ya/Yc-containing GST are not the only GST subunits shown to be capable of this 'suicidal' defence mechanism. The Yb subunits from rat liver have been implicated in the covalent binding of ethacrynic acid (Yamada and Kaplowitz, 1980).

It has thus been speculated that GST function in three separate ways to protect against chemically induced hepatocarcinogenesis.

1. The direct detoxification of the carcinogenic insult.
2. The binding of excess adrenal or gonadal steroid hormones which may affect the activation of carcinogens.
3. The sacrificial covalent binding of the carcinogen to facilitate its excretion.

1.10 : Measurement of GST B_1 as a sensitive marker of liver damage

The investigation of patients with suspected liver disease depends not only on a careful case history, physical examination but also on a number of laboratory tests. The use of ultrasound or radiological investigation may also be required and in a proportion of cases a liver biopsy may also be performed for histological examination. The standard biochemical tests used often involve a test of the structural integrity of the liver (serum transaminase), its ability to transport anions from blood to bile (bilirubin and bile salt concentrations) a measurement of cholestasis (alkaline phosphatase and γ-glutamyl transferase) and a test of hepatic protein synthesis (serum albumin concentrations and prothrombin time). Collectively these measurements are often referred to as 'liver function tests' although they do not all assess actual hepatic function and should more correctly be referred to as markers of liver damage. Also, many of these tests are not specific and may be abnormal in a variety of pathological conditions not affecting the liver. In addition to these commonly employed tests many others have been advocated, often for specific conditions examples being, caeruloplasmin for Wilsons disease, α-fetoprotein for hepatocarcinoma and α-1-antitrypsin in inherited disorders.

Following tissue damage enzymes and proteins are released from the cell into plasma and the measurement of the catalytic activity of intracellular enzymes after they have been released into the peripheral circulation is thus an established technique for assessing tissue damage in clinical enzymology. Characteristics required by an enzyme to function as a good biochemical marker include; tissue specificity, high cell to plasma ratio, ease of measurement and a suitable half life in the blood stream.

Properties that make the measurement of GST B_1 subunits by RIA an attractive candidate for assessing hepatic integrity include:

1) The immunohistochemistry of human liver has shown alpha GST to be uniformly distributed across the liver hepatocytes (Hiley *et al.*, 1988), whereas the aminotransferases are mainly periportal in location.

2) Its high concentration in human liver cytosol. As reported in Section 6 of this thesis, GST B_1 concentrations of up to 3% of the total cytosolic protein were measured.

3) High organ specificity. In the adult liver GST B_1 is normally expressed at around 5-fold greater concentrations than the level found in kidney, the kidney being the only other organ to express significant amounts of alpha

GST, although other tissues do express low levels of these isoenzymes (see Section 6).

4) The GST are not membrane bound and have a low molecular mass (52 000 daltons), therefore can be readily released into the circulation.

5) The short half-life of alpha GST in plasma. This property was recognised by Sherman *et al.* (1983a) who used RIA measurements of 'ligandin' (probably total GST B_1 and GST B_2) to monitor the changes in liver function that occurred in 68 patients with acute viral hepatitis. Sherman *et al.* (1983a) concluded that the short-half life of GST in plasma weakens its use as a diagnostic test in acute viral hepatitis. However, because of its short half-life (approximately 1 h) the measurement of GST B_1 or GST B_2 provides useful information concerning the progress of active disease and makes it an ideal marker for acute hepatocellular damage.

6) Alpha GST levels correlate well with liver histology in chronic active hepatitis, whereas AST activities show no such correlation (Sherman *et al.*, 1983a).

As an alternative approach to measuring catalytic activity, mass measurement by immunological techniques such as RIA or enzyme immunoassay may be used. Both methods have theoretical advantages and disadvantages. Enzymic activity measurements are generally rapid and convenient to perform on automated equipment, such as centrifugal analyzers, and if the appropriate assay conditions are selected considerable sensitivity may be achieved. However, activity may be decreased by the presence in plasma of circulating inhibitors, and differentiation between different isoenzymes may be difficult. Immunoassay methods for the measurement of the enzyme concentration are not usually affected by the presence of endogenous inhibitors or activators and if suitable antisera are available immunologically distinct isoenzymes can be measured, thus providing potentially improved tissue specificity for the test. Unfortunately, immunoassay techniques are usually slow and labour intensive since they do not readily lend themselves to automation. Both activity and immunoassay methods have been reported for the estimation of GST levels in plasma or serum.

The first substrates, used to determine GST activity in rat tissue, included 1,2-dichloro-4-nitrobenzene (Booth *et al.*, 1961) and bromsulphthalein (Coombes & Stakelum, 1961). These two compounds were substrates for rat Yb subunits but could

not act as substrates for most other GST subunits including the human alpha-class found in liver. With the introduction of CDNB, a substrate that is active with most forms of GST (Clark *et al.*, 1973), the measurement of GST activity in human serum became an attractive possibility as a sensitive index of liver integrity, especially after Kamisaka *et al.* (1975) demonstrated that up to 3% of human liver cytosolic protein was composed of GST. However even with CDNB, it was difficult to obtain sufficient sensitivity to permit precise measurement of the activity of GST in plasma or serum.

Measurement of GST activity is susceptible to two main sources of error. Firstly, there are various non-substrate ligands that bind to GST and inhibit enzymic activity; physiologically important examples are bile salts (see section 10) and bilirubin (Simons and Vander Jagt, 1980). The concentrations of these ligands in plasma are often elevated in liver disease and, as a consequence, they may suppress GST activity to levels which lie within the reference range. Secondly, platelets, erythrocytes, white cells, muscle, lung and many other tissues all contain significant quantities of GST pi which may leak into plasma in health and in a variety of non-hepatic diseases. GST pi not only reacts with CDNB but it does so with a higher specific activity than that of the alpha-class GST (Stockman *et al.*, 1987). Thus if CDNB is used as a substrate, poor organ specificity results. Haemoglobin released from erythrocytes inhibits GST activity with CDNB (Harvey and Beutler, 1982) and also interferes with monitoring the colorimetric reaction therefore, haemolysis invalidates GST activity measurements. Despite all these potential problems a study on GST activity in human serum has been reported (Adachi *et al.*, 1980), however, the results were disappointing, with sensitivity appearing to be the main problem. Adachi *et al.* (1980) found in acute hepatitis abnormal serum GST activity could only be found when ALT activity exceed 5 times the upper reference limit.

The introduction of immunoassay methods for the determination of specific GST isoenzymes in plasma has resulted in an improved sensitivity over the enzymatic methods. The first report of a RIA for GST was that of Bass *et al.* (1977); these workers developed an assay for measuring rat 'ligandin' (alpha-class GST). The RIA was used initially to estimate the tissue distribution of 'ligandin in the rat and the changes that occurred in tissue after the rats had been treated with phenobarbital. Shortly after, the same group used the RIA to measure the concentration of 'ligandin' in rat plasma following the production of hepatocellular necrosis by carbon tetrachloride (Bass *et al.*, 1978). The workers found that plasma 'ligandin' increased at a greater rate and to higher levels than aspartate aminotransferase (AST) activity following the

administration of carbon tetrachloride or after bile duct ligation. In both situations the plasma 'ligandin' returned to normal levels when AST remained elevated.

The first RIA for human 'ligandin' was developed by Tsuru *et al.* (1978) who found elevated concentrations of the protein in a variety of liver disorders. However, there was still considerable overlap between concentrations measured in controls and in patients with liver disease. The method of Tsuru *et al.* (1978) showed poor sensitivity, and it was not possible to define the lower reference limit; the upper limit of the reference range was 5.3 μg/L. Sherman *et al.* (1983a, 1983b) described an RIA method for human 'ligandin' that allowed measurement in normal plasma, but this too was not sufficiently sensitive to define its lower limit of the reference range; the upper limit for the reference was 12 μg/L. Subsequently assays with good sensitivity and precision were described for both GST B_1 and GST B_2 subunits (Beckett and Hayes, 1984).

The assays described for GST B_1 and GST B_2 by Beckett and Hayes (1984) had two main advantages over previous immunoassays. Firstly, Beckett and Hayes had available antisera which were able to differentiate between the different basic subunits, GST B_1 and GST B_2, previous assays for 'ligandin' probably measured both subunits. Secondly, Beckett and Hayes (1984) employed the technique of delayed tracer addition, which resulted in improved sensitivity of approximately three-fold. The reference range quoted for GST B_1 in 28 healthy volunteer subjects was 1.2-4.1 μg/L while, the GST B_2 reference range was 0.6-1.8 μg/L and for the first time a significant correlation (r = 0.80) was described between alanine aminotransferase (ALT) levels and GST B_1 concentrations in normal subjects. Two studies have shown the measurement of plasma GST B_1 isoenzyme to be more sensitive in assessing hepatic damage compared to the measurement of the GST B_2 isoenzyme (Beckett *et al.*, 1987 & 1989a) and in no study where both isoenzymes have been measured have the GST B_2 levels proved to be more informative than the GST B_1 levels. This is probably accounted for by the fact that the GST B_1 isoenzyme is the major alpha-class GST expressed in majority of adult livers. Therefore, in this thesis only plasma GST B_1 was measured as a test of liver damage.

Although the measurement of GST levels by RIA have advantages over enzymatic methods as far as sensitivity is concerned the time taken to perform a RIA (about 40 h) is considerable compared with the time taken for an enzyme measurement of ALT or total GST activity (about 15 min). Enzyme immunoassay methods, utilising fluorescent labelled substrates, that could be performed in one day

for anionic and cationic GST have been described (Hirano *et al.*, 1984) but, their precision and sensitivity was unsatisfactory.

1.11 : Plasma GST B_1 in acute liver damage

Beckett *et al.* (1985a, 1985b) have monitored patients admitted with paracetamol poisoning to compare plasma levels of GST B_1, GST B_2, ALT and AST levels in acute drug-induced liver damage. In these studies of paracetamol-induced liver damage Beckett *et al.* (1985a, 1985b), demonstrated the extreme sensitivity of GST B_1 or GST B_2 measurements for the detection of acute cellular damage. Seventeen of the 18 patients they studied had abnormal GST concentrations while in contrast only seven patients had abnormal plasma activities of ALT or AST. Beckett *et al.* (1989b) compared the sensitivity of GST B_1, F protein and ALT measurements for detection of liver damage in patients with paracetamol overdose and concluded that GST B_1 and F protein offered clear advantages over ALT for detecting minor degrees of acute liver dysfunction, particularly when only centrilobular damage may be involved.

Other situations where plasma GST concentrations have been measured and found to be of value include: changes in plasma GST B_1 after alcohol ingestion (Hayes *et al.*, 1990), assessing liver damage in neonates who suffered birth asphyxia (Beckett *et al.*, 1989a) and assessing subclinical liver damage after halothane anaesthesia (Hussey *et al.*, 1988; Allan *et al.*, 1987).

The data described above all indicate that plasma GST B_1 concentrations provide a more sensitive index of hepatic integrity than the aminotransferases. Sherman *et al.* (1983a) also concluded that the RIA measurement of 'basic ' GST in serum was a good prognostic marker in the investigation of viral hepatitis, although considered that the short half-life of 'basic' GST in plasma weakened its use as a diagnostic test. Since the AST level had to be elevated by definition in every case of acute viral hepatitis in the study by Sherman *et al.* (1983a), they were obviously limited to finding increased serum levels of 'basic' GST in only as many or in fewer patients. Possibly if different criteria had been employed to assess the hepatitis 'basic' GST would have been elevated in the early stages of the disease whereas, AST values may well still have been within their reference range.

1.12 : Measurement of GST B_1 in chronic liver disease

In chronic hepatitis markers used to assess disease activity include, aminotransferase activity, serum globulin concentrations, clinical symptoms and histology. Biopsy

appearance is the 'gold standard' by which all other tests are compared. Since biopsy cannot be repeated frequently the aminotransferases are the most commonly used test for day to day monitoring, even although it is well established that correlation of AST and ALT with disease activity is poor (Clermont and Chalmers, 1967). Sherman *et al.* (1983a) showed that serum GST levels correlated significantly ($P<0.01$) with the activity of the hepatitis, as assessed by histology, whereas AST activities showed no such correlation. Larger studies have never been reported to confirm these findings, but the data indicates that plasma GST B_1 measurements may provide an alternative to liver biopsy in monitoring the progress of chronic active hepatitis and its response to treatment.

Elevated plasma concentrations of alpha GST have been reported in more than 50% of patients with cirrhosis, in three separate studies (Beckett *et al.*, 1987; Tsuru *et al.*, 1978; Hirano *et al.*, 1984). Sherman *et al.* (1983a) however, reported normal 'basic' GST levels in all 20 patients with cirrhosis, all of whom had raised plasma AST activities. The data of Beckett *et al.* (1987) who studied plasma GST B_1, GST B_2 and AST levels in 79 alcoholic cirrhotic patients, indicates that measurements of either GST B_1 or GST B_2 provides information that is different and additional to, the information obtained from AST activities alone. Not all patients who had raised AST activities had raised GST B_1 concentrations and vice versa. Abnormal results were detected in 53 of the 79 cirrhotic patients, using both measurements, whereas abnormal AST activities were only evident in 35 patients. The reason for the discrepancy between GST B_1 and AST levels in certain patients in not known, but lobular distribution, length of plasma half-life and differing sensitivities are probable explanations. It is also possible that GST synthesis maybe induced or even down-regulated in certain individuals. Preliminary immunohistochemical work would appear to indicate that in alcoholic cirrhotic livers the GST pi isoenzyme (not normally expressed in adult hepatocytes) is expressed (Personal communication Dr. D. Harrison).

Before the advent of effective treatment for hyperthyroidism, hepatobiliary complications were commonly associated with this disease. As prolonged severe hyperthyroidism now occurs only rarely, severe hepatobiliary dysfunction associated with hyperthyroidism is seldom encountered. However, Beckett *et al.* (1985c) reported abnormal GST B_1 concentrations in ten out of 14 hyperthyroid patients at presentation, whose GST B_1 levels returned to normal after treatment and concluded that hyperthyroidism may produce subclinical liver damage in a high proportion of patients. There is little evidence to suggest that hypothyroidism affects liver function, but

patients subsequently receive thyroxine replacement therapy and Beckett *et al.* (1985c), in eight newly diagnosed hypothyroid patients, demonstrated that these patients had normal GST B_1 concentrations at presentation but showed a significant increase in GST B_1 levels after thyroxine replacement therapy. Similar though less consistent changes were seen in the results of AST, γGT and ALP. It was concluded that hypothyroid patients receiving thyroxine replacement therapy may suffer subclinical liver damage as a result of their therapy and careful monitoring of thyroid stimulating hormone was expedient (Gow *et al.* 1987). Periodic biochemical assessment of liver function was also advised.

The mechanism by which thyroxine increases plasma GST B_1, although not known with certainty, is thought to be hepatic damage as opposed to hepatic induction of the enzyme. Working with a rat model, where the rats were administered high doses of thyroxine, high levels of plasma YaYa GST (rat equivalent of human GST B_1) were observed with concomitant rises in ALT activity. Thyroxine administration also produced a significant fall in the hepatic content of YaYa GST (Beckett *et al.*, 1986).

1.13 : Measurement of 'ligandin' in human hepatocellular carcinoma

Ohmi and Arias (1981), showed that 11 out of 15 patients with primary hepatocellular carcinomas had elevated serum ligandin levels but only three out of 22 patients with extensive hepatic metastases had elevated serum ligandin levels. Sherman *et al.* (1983b) reported elevated levels of 'ligandin' in 17 out of 55 patients diagnosed as having hepatocellular carcinoma. Both groups reported that hepatic tissue levels of 'ligandin' decreased. The mechanism whereby increased serum 'ligandin' concentrations occur in primary hepatocellular carcinoma is not known but Ohmi and Arias (1981) showed by transplanting rat hepatoma cells into nude mice that serum 'ligandin' rises as the tumour grows and concluded that the tumour was the source of the 'ligandin'. In contrast, Sherman *et al.* (1983b) demonstrated that, although elevated serum 'ligandin' concentrations were seen in some patients there was no correlation with the intensity of tumour staining for 'ligandin' and postulated that serum 'ligandin' originated from normal adjacent cells damaged by tumour growth.

1.14 : Measurement of 'ligandin' in urine as an indicator of renal damage

After Kirsch *et al.* (1975) published the paper 'Structural and functional studies of ligandin, a major renal organic anion-binding protein' (work done with rat tissue), a number of studies were reported concerning the measurement of ligandin in rat urine

as a marker for renal damage. Ligandinuria was shown to occur in rats with acute tubular necrosis (Feinfeld *et al.*, 1977) and in rats with gentamicin-induced nephrotoxicity (Feinfeld *et al.*, 1981).

In 1980, Campbell *et al.* using immunohistological techniques localized the expression of 'ligandin' in human kidney to the proximal convoluted tubule and the thick segment of Henle's loop. These findings have been supported by recent immunohistochemical work where, alpha GST was reported to be present in the proximal convoluted tubules with little staining elsewhere, whereas staining for GST pi was weak in the proximal tubules but strong in the in the distal convoluted tubules and medullary tubules. Staining for mu-class and microsomal GST was also reported (Harrison *et al.*, 1989).

Feinfeld *et al.* (1978) looked for 'ligandin' in perfusates from human kidneys that were stored prior to transplantation into uraemic patients and reported that perfusates from 8 kidneys developing post-transplantation acute tubular necrosis had immunodetectable 'ligandin' levels whereas, the perfusates of 5 kidneys which functioned immediately where negative for ligandin. This study was confirmed by Cho *et al.* (1981) on a larger sample number, the inference being that the appearance of 'ligandin' in the perfusate of a kidney preserved for transplantation could be used to detect significant ischemic tubular necrosis and predict acute renal failure following transplantation.

Backman *et al.* (1989) monitored, by radioimmunoassay, the levels of 'basic' GST in the urine of 69 recipients of renal allografts. Patients receiving Cyclosporin A without toxicity or rejection did not express 'basic' GST in their urine whereas the urine of patients with Cyclosporin A-induced nephrotoxicity contained significant amounts of GST. However, patients with allograft rejection also showed increased urinary concentrations of the 'basic' GST although lower values than the Cyclosporin A-induced nephrotoxicity. Measurement of both GST pi and GST B_1 in urine may have given a better differentiation between the two conditions. If the urinary excretion of GST B_1 is high as a result of renal injury, and GST pi excretion is low, then it is likely that damage is restricted to the proximal convoluted tubule and would be due to Cyclosporin toxicity (Cyclosporin is thought to be primarily a proximal tubule toxin). The presence of both GST pi and GST B_1 in urine may reflect more widespread tubular and even glomerular damage therefore may indicate transplant rejection.

1.15 : Measurement of GST pi in human tissues

The first measurements of GST pi were probably performed by Scott and Wright (1980) who measured erythrocyte GST 1-chloro-2,4-dinitrobenzene conjugating activity in a large number of blood donors. A comparable study was performed by Strange *et al.* (1982) who also looked at GST activity in erythrocytes although they first semi-purified the enzyme to eliminate haemoglobin interference. These studies were performed to determine the normal expression of GST in erythrocytes, whereas a report by Kilipikari and Savolainen (1984) found a decrease in erythrocyte GST activity in workers exposed to hot rubber fumes as compared to normals. Ansari *et al.* (1987) proposed measuring GST activity in erythrocytes (GST pi being the predominant GST isoenzyme present in these cells) as a possible marker for chemical exposure to industrial toxicants, such as acrolein, propylene oxide, ethylene dibromide and ethylene dichloride, all of which demonstrated dose-dependent inactivation of human erythrocyte GST, but no subsequent reports have appeared from these workers.

After GST pi was purified and antibodies became available, the technique of immunohistochemistry demonstrated that GST pi was elevated in tumour compared to normal tissue in a number of human tumours originating from colon (Kodate *et al.*, 1986), stomach (Tsutsumi *et al.*, 1987) and lung (Eimoto *et al.*, 1988). It was therefore suggested that GST pi may be a useful tumour marker. The first immunoassay for the quantification of GST pi was an enzyme immunoassay described by Hirano *et al.* (1984) for anionic GST but this assay had poor precision and sensitivity. Niitsu *et al.* (1989) and Tsuchida *et al.* (1989) have both recently described immunoassays where they advocate that the measurement of GST pi levels in serum is a useful tumour marker for gastrointestinal malignancies. Yoshizaki *et al.* (1989) described a radioimmunoassay for erythrocyte acidic GST and used it to measure serum GST pi concentrations in various haematological conditions, and suggested that GST pi may be of use as a clinical marker of increased destruction and/or overproduction of blood cells such as paroxysmal nocturnal haemoglobinuria and acute lymphocytic leukaemia.

Measurement of GST pi mRNA using a cDNA probe has also been described (Moscow *et al.* 1988 & 1989). These workers identified an inverse relationship of GST pi mRNA levels with oestrogen receptor status in breast cancer tissue and described elevated levels of GST pi mRNA in tumour as compared to normal tissue in various cancers.

1.16 : Measurement of mu-class GST in human tissues

Polymorphism of the mu-class GST, GST μ and GST ψ have been examined by three separate methods; zymogram analysis on starch gels (Board, 1981; Harada *et al.*, 1987) specific catalytic activity towards *trans*-stilbene oxide (Seidegard *et al.*, 1986 & 1987) and radioimmunoassay (Hussey *et al.*, 1987a). The subject of GST μ expression and possible susceptibility to disease states in individuals lacking the enzyme is discussed more fully in Section 7.

1.17 : Aims of the thesis

The aims of the thesis were to;

1) Purify the GST pi form of cytosolic GST from human placenta, lung and erythrocyte.
2) Raise antibodies against pure GST pi antigen and determine if GST pi from placenta, lung and erythrocyte was immunologically identical.
3) Develop a sensitive and specific immunoassay for GST pi quantification.
4) To use the radioimmunoassay developed for GST pi and the existing radioimmunoassays for the alpha and mu-class GST to investigate:
   a) The clinical utility of GST pi measurements as a tumour marker.
   b) Expression of GST isoenzymes in breast cancer cytosols.
   c) Expression of GST isoenzymes in adult normal and tumour tissues and tissues.
   d) Expression of GST μ in lung cancer patients and age matched controls.
   e) Developmental aspects of GST in normal tissues.
   f) Occurrence of GST isoenzymes in lung lavage and bile.
   g) Liver damage after general anaesthesia and hypoglycaemia

Section 2 : MATERIALS AND GENERAL METHODS

2.01 : Equipment and Chemical Suppliers

Abbott Diagnostics Division, Maidenhead, Berks., UK
'Thrombotect' blood collection tubes; Platelet Factor 4 RIA kit.

Amersham International, Amersham, Bucks., UK
^{125}I-labelled sodium iodide.

BDH Chemicals Ltd., Poole, Dorset, UK
Acetic acid; acrylamide; 2-amino-2-(hydroxymethyl)propane-1,3-diol(Tris); ammonium persulphate; brij-35 (30% solution); bromophenol blue; butan-1-ol; calcium chloride; cellulose microcrystalline; chloramine T; 1-chloro-2,4-dinitrobenzene; L-cysteine; dipotassium hydrogen orthophosphate; disodium hydrogen orthophosphate; ethanol; ethanolamine; ethylenediaminetetra-acetic acid (EDTA); formaldehyde; glycerol; glycine; hydrochloric acid; hydrogen peroxide; 2-mercaptoethanol; methanol; nitric acid; NN'-methylenebisacrylamide; orthophosphoric acid; potassium chloride; potassium dihydrogen orthophosphate; potassium dichromate; potassium hydroxide; potassium iodide; silver nitrate; sodium acetate; sodium borate; sodium carbonate; sodium chloride; sodium dihydrogen orthophosphate; sodium dodecyl sulphate; sodium hydroxide; sucrose; NNN'N'-tetramethyl ethylenediamine (TEMED); trichloroacetic acid; Whatman No. 1 filter paper.

Bio-Rad Laboratories, Watford, Herts., UK
Nitrocellulose paper; immuno-blot assay kit; Tween-20.

Koch-Light Laboratories Ltd., Colinbrook, Berks., UK
Cumene hydroperoxide.

L.I.P. (Equipment & Services) Ltd., Shipley, West Yorkshire, UK
12 x 75 mm RIA tubes.

LKB-Products, Bromma, Sweden
Ampholine solutions.

Millipore (UK) Ltd., Harrow, Middlesex, UK
Ultrafiltration membranes.

Pharmacia Fine Chemicals, Uppsala, Sweden
Isoelectric focusing protein standards.

Randox Laboratories, Crumlin, Northern Ireland
Commercial kits for the estimation of: alanine aminotransferase (ALT), aspartate aminotransferase (AST) and γ-glutamyl transferase (γGT) activities.

Scientific Instruments Centre Ltd., London, UK
Visking tubing for dialysis.

Scottish Antibody Production Unit, Carluke, Lanarkshire, UK
Normal rabbit serum (NRS) and anti-rabbit IgG (donkey) (DARS) precipitating serum.

Sigma Chemical Co. (London) Ltd., Poole, Dorset, UK
Bovine serum albumin (BSA); Coomassie Brilliant Blue (R-250 and G-250); histidine; Freund's adjuvant (complete and incomplete); gelatin; glutathione reductase; glutathione (reduced form); nicotinamide adenine dinucleotide phosphate (reduced form, type X); Pharmalyte pH 3-10; Polybuffer 74; sodium glycocholate; sodium taurochenodeoxycholate.

2.02 : Materials for chromatography

Pharmacia (Laboratory Separation Division), Milton Keynes, Bucks., UK
Mono Q HR 5/5 Fast Protein Liquid Chromatography (FPLC) anion exchange column; Polybuffer exchanger (PBE) 96.

Sigma Chemical Co., (London) Ltd., Poole, Dorset, UK
Epoxy-activated Sepharose 6B; Sephadex G-25; Sephadex G-100; SP-Sephadex.

2.03 : Materials not obtained from commercial sources

S-Hexylglutathione and S-hexylglutathione-Sepharose 6B affinity matrix was a gift from Dr.J.D.Hayes. Glutathione-Sepharose 6B affinity matrix was prepared by coupling the

sulphydryl moiety of glutathione to the epoxy-activated Sepharose 6B as described by Simons & Vander Jagt (1977).

Antisera raised against human alpha GST, B_1 and B_2 and GST μ, as well as the corresponding standards, for RIA, were provided by Dr J.D. Hayes and Dr G.J. Beckett. Affinity-purified cytosolic rat lung GST was also provided by Dr J.D. Hayes. Purified rat and mouse Y_f GST, used for cross-reactivity experiments, was a gift from Dr L.M. McLellan. Purified GST4 from human heart tissue was a gift from Dr R.C. Strange.

2.04 : Animals

New Zealand white rabbits, used for raising antisera, were obtained from Charles River Breeding Laboratories, UK.

2.05 : Protein determination

Protein concentrations, in fraction samples eluted from chromatography columns, were calculated from the extinction values at 280 nm. An absorbance reading of 1.0 was taken to represent a protein concentration of 1 g/L.

All other protein estimations were carried out utilizing the dye-binding technique of Bradford (1976) adapted for use on a Cobas Fara (Roche Diagnostics, Welwyn Garden City, UK) centrifugal analyser. Working Coomassie Brilliant Blue G-250 was prepared as described by Bradford (1976) although it was filtered, before use, through Whatman grade 1 filter paper. Reagent (256 μl) was added to each cuvette and incubated at 37 °C for 100 s with an initial absorbance reading (595 nm) taken at 95 s, after the addition of 25 μl of sample plus 50 μl of distilled water as diluent, the cuvettes were incubated at 37 °C for a further 180 s, when a final absorbance reading was taken at 595 nm. A standard curve, using bovine serum albumin, was constructed from 0 to 100 mg/l. The difference between the final and initial absorbance points was calculated and a standard curve was plotted. The protein concentration of unknown samples were interpolated from this curve. Samples with protein concentrations greater than 100 mg/l were diluted in distilled water. The within batch CV was found to be <2% and the between bath CV was <5%.

2.06 : Sodium determination

Sodium concentrations, in the Fast Protein Liquid Chromatography (FPLC) column eluates, were measured by flame photometry using an IL543 photometer (Instrumentation Laboratory (UK) Ltd., Warrington, Cheshire, UK).

2.07 : GST activity

Assays for cytosolic GST activity with 1-chloro-2,4-dinitrobenzene (CDNB) as substrate were carried on the Cobas Fara centrifugal analyser essentially as described by Hayes & Clarkson (1982). All assays were carried out at 37 °C and a non-enzymic rate determined which was subtracted from the rates observed in samples. Up to 29 samples were simultaneously pre-incubated with reduced GSH and the reactions started with the addition of CDNB. The final substrate concentrations were 2 mmol and 1 mmol for GSH and CDNB respectively in a reaction volume of 250 μl containing 0.1 mol sodium phosphate buffer pH 6.5. The reactions were monitored by initial measurement of absorbance at 340 nm, 10 s after mixing, followed by 7 further absorbance measurements at 5 s intervals. Reaction rates were determined by an integral kinetic data analysis program, which performed linear regression analysis on the 8 absorbance readings from each cuvette. The ΔA/min was multiplied by a conversion factor, which depended on the sample volume, and the results expressed as ΔA/min/mL of sample. The assay was linear up to levels of 300 ΔA/min/mL, samples with enzyme activity >300 were diluted in distilled water and re-assayed. The within batch CV was <4% and between batch CV <10%. All samples were performed in duplicate.

2.08 : Glutathione Peroxidase Activity

Measurement of selenium dependent (sGPX) and independent (tGPX) glutathione peroxidase utilised an adaptation of the Paglia and Valentine (1967) method for use on a Cobas Fara centrifugal analyser. The final concentration of the peroxides was 0.6 mmol/L for hydrogen peroxide and 1.2 mmol/L for cumene hydroperoxide. The assay solution contained 60 mmol/L Tris/HCl buffer pH 7.6 (21 °C), 0.12 mmol/L EDTA, 1 mmol/L NaN_3, 0.33 mmol/L NADPH, 1.3 mmol/L reduced glutathione and 1.3 units/mL glutathione reductase. After a 5 minute incubation (20 μL sample: 50 μL H_2O wash: 260 μL assay solution) the reaction was started with the addition of peroxide (5 μL). A water blank value was subtracted for each determination. The rate of the reaction was determined by following the decrease in absorbance at 340nm at 37 °C. Results were expressed as umol of NADPH oxidised/min/L. The within-batch CV was <4% and the between batch CV was <10%. All samples were performed in duplicate.

2.09 : SDS/Polyacrylamide-gel electrophoresis (SDS/PAGE)

SDS/PAGE was carried out in slab gels (0.1 cm x 16.5 cm x 18 cm) at room temperature using a PROTEAN II electrophoresis system (Bio-Rad Laboratories Ltd., Watford, Herts., UK) in the presence of 1% SDS (w/v) and employing the buffer system described by Laemmli (1970). The "resolving gel" was 14 cm long, of 12% (w/v) polyacrylamide and 2.6% (w/v) NN'-methylenebisacrylamide in 375 mmol/L Tris/HCl buffer pH 8.8. The "stacking gel" was 2.5 cm long and comprised 3% (w/v) polyacrylamide in a 125 mmol/L Tris/HCl buffer pH 6.8 and was formed on top of the "resolving gel".

Protein samples were prepared for electrophoresis by heating at 90 °C for 10 min in an aqueous solution of: SDS, 35 mmol/L; glycerol, 1.4 mmol/L; 2-mercaptoethanol, 0.3 mmol/L and bromophenol blue, 15 mmol/L. The desired volume of these "boiling mixes" were applied to wells in the "stacking gel" and electrophoresis was carried out, at 50 mA per gel , through the "stacking gel" and then at 25 mA per gel, through the "resolving gel". The current was stopped when the bromophenol blue marker reached within 0.5 cm from the end of the gel. The gel was stained either with a 0.2% (w/v) Coomassie Brilliant Blue R in water/methanol/acetic acid (50:50:7, by vol.) and destained in several changes of water/methanol/acetic acid (88:5:7 by vol.) or by the highly sensitive silver stain technique as described by Switzer *et al.* (1979).

The silver stain technique involved soaking the gel for at least 1 h in a water/methanol/acetic acid (38:50:12, by vol.) solution before oxidising for 15 min with 3.4 mmol/L potassium dichromate containing 3.2 mmol/L nitric acid. The gel was then washed for 10 min in distilled water before being placed in the silver stain consisting of 12 mmol/L silver nitrate for 20 min, after a brief rinse in distilled water the gel was placed in the developing solution consisting of 6.7 mmol/L formaldehyde in 280 mmol/L sodium carbonate for 5-10 min and the reaction stopped by the addition 3% (w/v) acetic acid solution.

Molecular mass values for subunits of cytosolic GST were estimated by using GST mixtures from rat, where subunit Mr values had previously established (Hayes & Mantle, 1986c).

2.10 : Isoelectric focusing in polyacrylamide-gels

Isoelectric focusing (IEF) was performed in 5% (w/v) polyacrylamide slab gels (24 cm x 11.5 cm x 0.2 cm), containing 10% glycerol (v/v) and 5% Carrier Ampholine mixture (v/v) pH range 3-10. Electrofocusing was carried out on a LKB 2117 Multiphor using a

LKB 2103 power supply as described by the manufacturer (LKB-Produkter AB, Bromma, Sweden). The electrode solutions were 1 mol H_3PO_4 (anode) and 1 mol NaOH (cathode). Samples (25 μg protein) were applied to strips (1 cm x 1 cm) of Whatman No. 1 paper placed 3 cm from the cathode electrode. Pharmacia IEF calibration markers were included on all gels and comprised; trypsinogen, pI 9.30; lentil lectin, pI, 8.65, 8.45 and 8.15; myoglobin, pI 7.35 and 6.85; human carbonic anhydrase B, pI 6.55; bovine carbonic anhydrase B, pI 5.85; β-lactoglobulin A, pI 5.20; soybean trypsin inhibitor, pI 4.55 and amyloglucosidase pI 3.50.

The focusing was performed at a constant power (25W) for 3 h, the sample applicators being removed after 1 h. The gels were fixed in an aqueous solution of sulphosalicylic acid (3.5%, w/v) and trichloroacetic acid (11.5%, w/v) for 30 min before washing for 30 min in destaining solution composed of, water/ethanol/acetic acid (67:25:8 by vol.). A 0.12% (w/v) Coomassie Brilliant Blue R solution was employed to stain the gel at 60 °C for 10 min. Destaining was achieved using several changes of destaining solution.

2.11 : Western blotting

Western blotting was performed by the method of Towbin *et al.* (1979), as described by Hayes & Mantle (1986a). Proteins were subject to SDS/PAGE and following electrophoresis, the gels washed in 25 mmol/L Tris containing 192 mmol/L glycine, pH 8.3 at 20 °C, for 20 to 30 min before being transferred to nitrocellulose paper. Transfer was carried out in the same buffer as above, using a Bio-Rad Trans-Blot cell and Bio-Rad model 250/2.5 power supply (Bio-Rad Laboratories, Watford, Herts., UK). The electrophoretic transfer of polypeptides to nitrocellulose paper was performed at 0.25 A for 4 h, at 10 °C. The paper was then soaked in a blocking solution composed of gelatin 3% (w/v) in 20 mmol/L Tris/HCl, pH 7.5, containing 500 mmol/L NaCl, for 16 h at 20 °C, to reduce the non-specific binding of antibody to the nitrocellulose. After blocking with gelatin, the nitrocellulose papers were incubated for 3 h at 20 °C with the first antibody which was diluted 1:3000 in 200 mL of 1% (w/v) gelatin in 20 mmol/L Tris/HCl buffer, pH 7.5, containing 500 mmol/L NaCl and 0.05% (v/v) Tween-20. Following washing of the papers with distilled water and buffer, visualisation of the polypeptides which cross-reacted with the specific first antibody was achieved using a second (goat anti-rabbit IgG) antibody labelled with horseradish-peroxidase (Bio-Rad immunoblot assay kit), according to the manufacturers instructions (Bio-Rad Laboratories, Watford, Herts., UK). A portion of the immunogen for the first antibody

was run in parallel with test proteins, to act as standard and control.

2.12 : Preparation of ^{125}I GST pi, B_1, B_2, and μ

The iodination procedure was identical for all four proteins. Protein (10 μL; 5 μg) and potassium phosphate buffer 0.25 mol/L pH 7.4 at 20 °C (10 μL) were added to a glass test tube followed by ^{125}I-sodium iodide (5 μL; 18.5 MBq) and chloramine T (10 μL; 16 μg). After 15 s cysteine (100 μL; 56 μg), potassium iodide (10 μg; 100 μg) and diluent (250 μL) were added to stop the reaction. The diluent consisted of 25 mmol/L potassium phosphate, pH 7.5 containing bovine serum albumin (1 g/L) and sodium azide (0.2 g/L). The contents of the tube were then transferred to a gel filtration column consisting of 1 cm x 12 cm bead of Sephadex G-25. The column had previously been equilibrated with diluent. The column was eluted with diluent (0.5 mL/min) and fractions containing iodinated protein pooled and used for the radioimmunoassay.

2.13 : Preparation of pre-precipitated DARS/NRS second antibody

This was prepared by mixing DARS (20 mL) with NRS (1.5 mL) and allowing precipitation to take place overnight at room temperature. After centrifugation (10 min 1500 x g) the supernatant was discarded, the precipitate washed twice with 20 mL assay diluent consisting of 25 mmol/L sodium phosphate, pH 7.5, bovine serum albumin (1 g/L) and sodium azide (0.2 g/L), and re-suspended in 125 mL assay diluent. This solution was stored at 4 °C.

2.14 : Radioimmunoassay of GST B_1, B_2 and μ.

These assays were performed essentially as described by Beckett & Hayes (1984) and Hussey *et al.* (1987). The assay protocols were identical for all 3 assays. All test reagents and specimens were brought to room temperature before beginning the test. Tubes were labelled for the performance of the test as follows:

a) Tubes 1 & 2, Total Count Tubes, for the determination of total radioactivity.

b) Tubes 3 & 4, Nonspecific Binding (NSB).

c) Tubes 5 to 20, for the appropriate standards in duplicate.

d) Tubes 21, etc. for the unknown specimens in duplicate.

Buffer, standard or sample (100 μL) was incubated with antiserum (100 μL), at 4 °C, for 24 h before tracer (100 μL; 40 000 cpm) was added to all tubes. The tubes were incubated for 24 h, at 4 °C, when DARS (100 μL) was added to all tubes, except 1 & 2,

and a further 1 h incubation at room temperature undertaken. A wash solution (2 mL; 0.05% aqueous Brij 35) was added to all tubes, except 1 & 2, and the tubes centrifuged for 30 min (4 °C, 3000 x g). The supernatant was decanted and the precipitate counted in a LKB 1261 Multigamma gamma counter and the data processing performed using the LKB 1224-Ria Calc. LM RIA evaluation program (LKB-Products, Bromma, Sweden).

Standards of GST were made up in assay diluent after the protein concentration had been determined using the method of Bradford (1976). Final, first antibody titres for GST B_1, B_2, and μ were 1 in 90 000, 1 in 45 000 and 1 in 3 000 respectively.

Section 3 : PURIFICATION AND CHARACTERISATION OF GST pi

3.01 : Purification of GST pi from human placenta

Normal 40-wk full-term placentas obtained within 8 h of delivery were used and all procedures were carried out either on ice or at 4 °C. The placenta (about 600g) was cut into 4 cm^3 pieces and, after the removal of the amniotic membrane, washed with 2 L of 0.25 mol/L sucrose solution to remove excess blood. The washed placenta (about 500 g) was homogenised with 1 litre 20 mmol/L sodium phosphate buffer pH 6.5 and centrifuged at 15 000 x g for 2 h. The supernatant (placental cytosol) was then dialysed for 24 h against two changes, each of 5 L of 20 mmol/L sodium phosphate buffer, pH 6.5. The cytosol was then applied to a 4.5 cm x 80 cm SP-Sephadex cation exchange column previously equilibrated with 20 mmol/L sodium phosphate buffer, pH 6.5. The flow-rate was 36 mL/h and 12 mL fractions were collected.

Glutathione S-transferase activity that was not retained by the column was combined (Pool 1) and applied immediately to an S-hexylglutathione-Sepharose 6B affinity column (1.6 cm x 17 cm). The column was washed overnight (24 mL/h) with 20 mmol/L Tris/HCl buffer, pH 7.8, containing 200 mmol/L NaCl. The GST enzymes were then eluted with 5 mmol/L S-hexylglutathione in the running buffer and 5 mL fractions were collected. The GST-containing fractions were combined (Pool 2) and dialysed against two changes, each of 2 L, of 25 mmol/L histidine/HCl buffer, pH 6.2, for 24 h.

The dialysed protein was subject to chromatofocusing which was performed using a 1.6 cm x 30 cm column of PBE 94 that was previously equilibrated with 25 mmol/L histidine/HCl buffer, pH 6.2; polybuffer 74 diluted 8-fold with distilled water and adjusted to pH 4.0 with HCl, was used as the elution buffer. Pool 2 was not loaded on to the chromatofocusing column until after 10 mL elution buffer had been applied, and the column was then eluted at 24 mL/h; 8 mL fractions were collected.
The peak of GST activity, which eluted in the region, pH 4.9-4.6, was combined (Pool 3) and re-applied to a S-hexylglutathione affinity column to remove polybuffer.
Following this second affinity chromatography step the GST was dialysed against two changes, each of 2 L, of 20 mmol/L sodium phosphate buffer, pH 6.5, and was stored at -70 °C until required.

3.02 : Purification of GST pi from human lung

Pieces of normal human lung were obtained within 8 h of death from the post-mortem suite. The GST pi from human lung was purified using a scheme identical to the human placenta purification described above.

3.03 : Purification of GST pi from human erythrocytes

Packed erythrocytes (250 mL) <6 h old, were obtained from whole blood sent to the Department of Clinical Chemistry for biochemical analysis. The blood was centrifuged at 2 500 x g for 15 min, and the plasma removed. The packed erythrocytes were washed twice in 20 mmol/L sodium phosphate buffer, pH 6.5, containing 110 mmol/L NaCl and filtered through cotton wool to minimise leucocyte contamination. The erythrocytes were harvested from the buffer by centrifugation at 2 500 x g and lysed by adding an equal volume of 20 mmol/L sodium phosphate buffer pH 6.5 (4 °C). The purification procedure of the erythrocyte GST was essentially the same as for the placental preparation except that the final affinity chromatography step was carried out using a glutathione affinity column (Simons and Vander Jagt, 1977) instead of a S-hexylglutathione affinity column.

The peak of GST activity, which eluted of the chromatofocusing column, in the region, pH 4.9-4.6, was combined and applied to a glutathione affinity column (1.6 cm x 17 cm) that had been equilibrated with 22 mmol/L sodium phosphate buffer, pH 7.0. The column was washed with the equilibration buffer overnight and the column developed with 50 mmol/L Tris/HCl (pH 9.6 at 4 °C) containing 5 mmol/L reduced glutathione. The peak of GST activity was dialysed against two changes, each of 2 L, of 20 mmol/L sodium phosphate buffer, pH 6.5, and stored at -70 °C until required.

3.04 : SDS/PAGE and IEF of purified GST pi

These techniques were carried out as described in the methods section (2.09 and 2.10). Both gels were stained using Coomassie Brilliant Blue. Approximately 5 μg of GST was applied to the SDS/page gel whereas 25 μg was applied to the IEF gel. Isoelectic points (pI) of the proteins were established by measuring the distance the standards had migrated from the cathode, plotting a graph of pI versus distance travelled and reading off the pI value for the unknowns.

3.05 : Results of GST pi Purification

Column profiles from each stage in the purification scheme for the placental preparation are shown in figures 3a-d. The column profiles for the lung and erythrocyte preparations were essentially the same as those illustrated for the placenta. The amount of pure GST pi recovered from placenta, lung and erythrocytes is shown in table 3a. The result of SDS/PAGE analysis is shown in figure 3e, all three pure GST proteins running as a single band with an apparent Mr weight of 24 800, when compared against rat lung affinity purified GST standards. The graph obtained from the standard proteins run during isoelectric focusing is illustrated in figure 3f, each GST was found to migrate as a single band with a pI of 4.75.

TABLE 3a: Comparison of human GST pi levels recovered from each tissue.

	Amount of Starting material	Amount of pure GST recovered	Yield of GST (% x 10^3)
Placenta	500 g	26.0 mg	5.2
Lung	734 g	16.1 mg	2.2
erythrocyte	250 mL Packed cells	1.2 mg	0.5

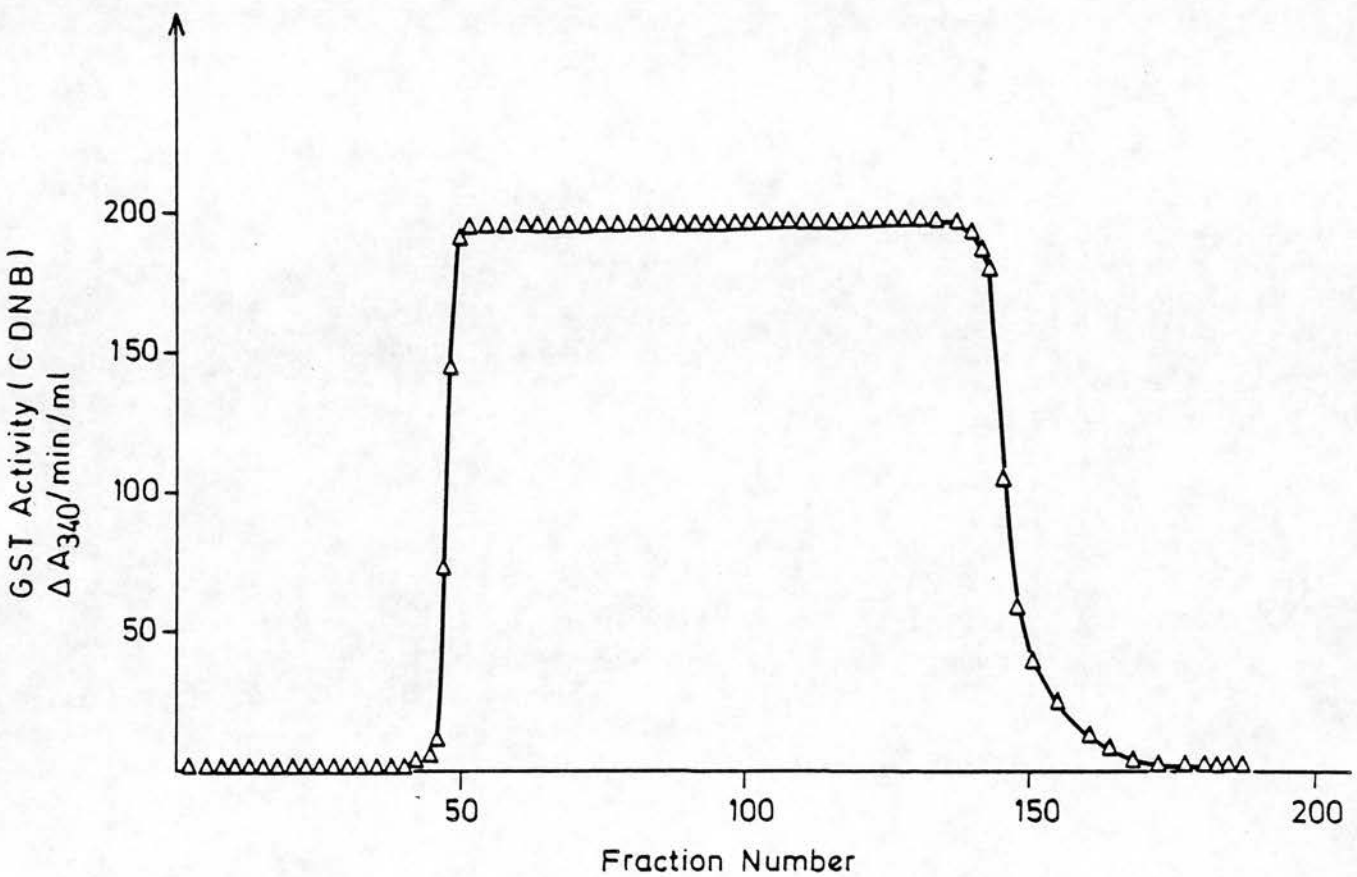

Figure 3a: Elution profile of placental GST from an SP-Sephadex cation exchange column. Placental cytosol was applied to the 4.5 cm x 80 cm column and the GST activity with CDNB (Δ) flowed straight through.

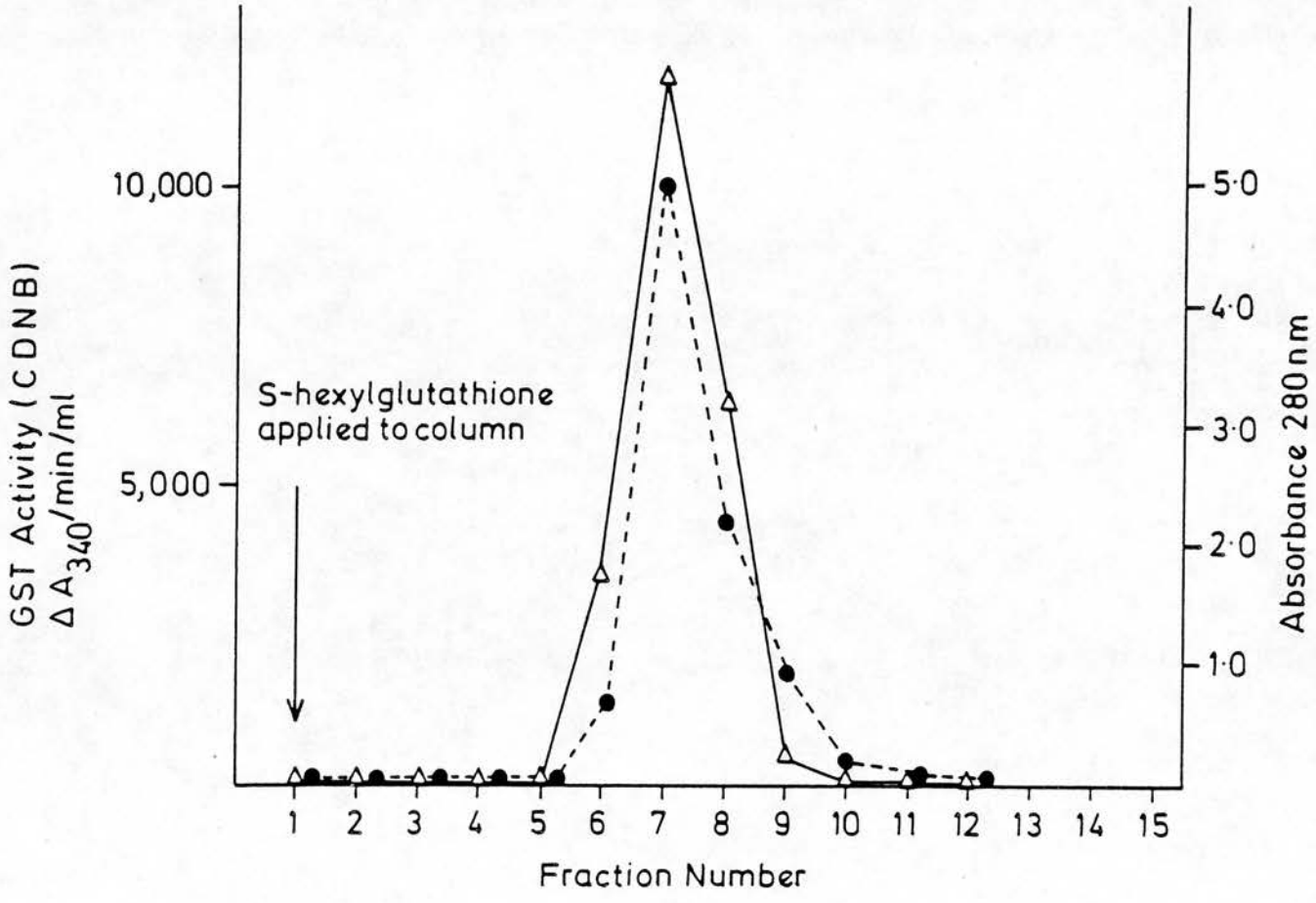

Figure 3b: Elution profile from the initial S-hexylglutathione affinity column step. Protein concentration and CDNB activity are represented by ● and Δ respectively.

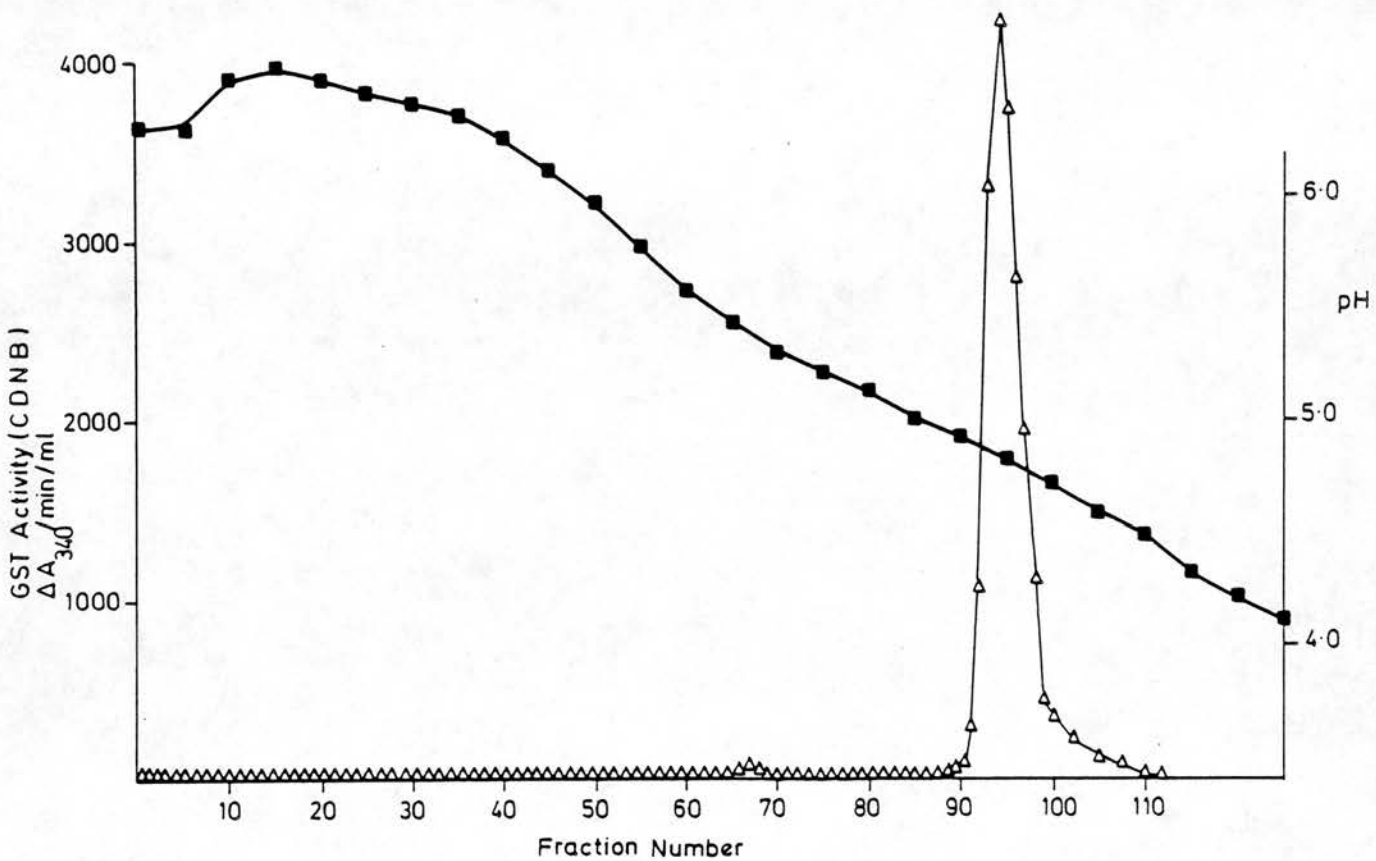

Figure 3c: Elution profile from the chromatofocusing column. The peak of GST activity with CDNB (Δ) eluted in the region of pH (■) 4.9-4.6.

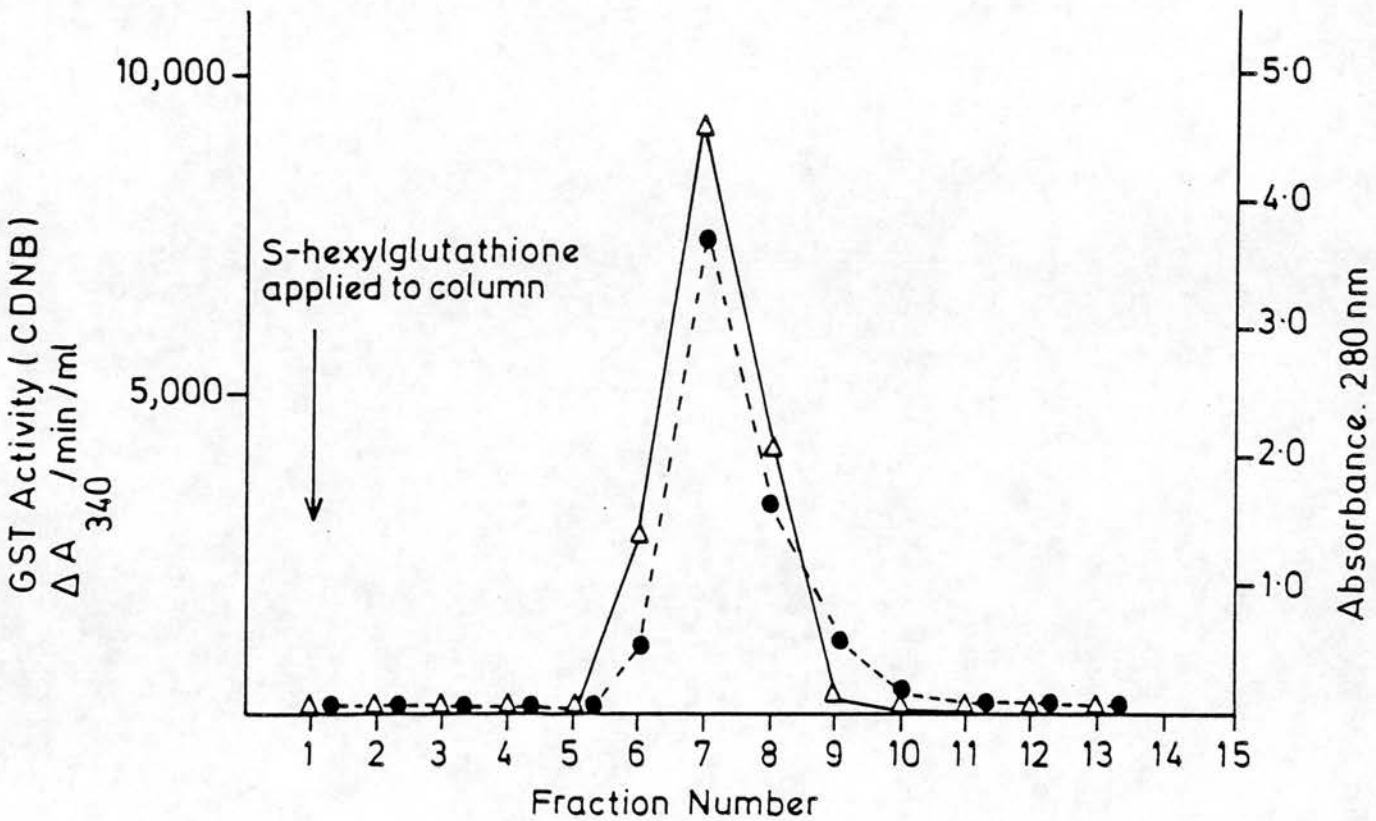

Figure 3d: Elution profile from the second S-hexylglutathione affinity column step. Protein concentration and CDNB activity are represented by ● and △ respectively.

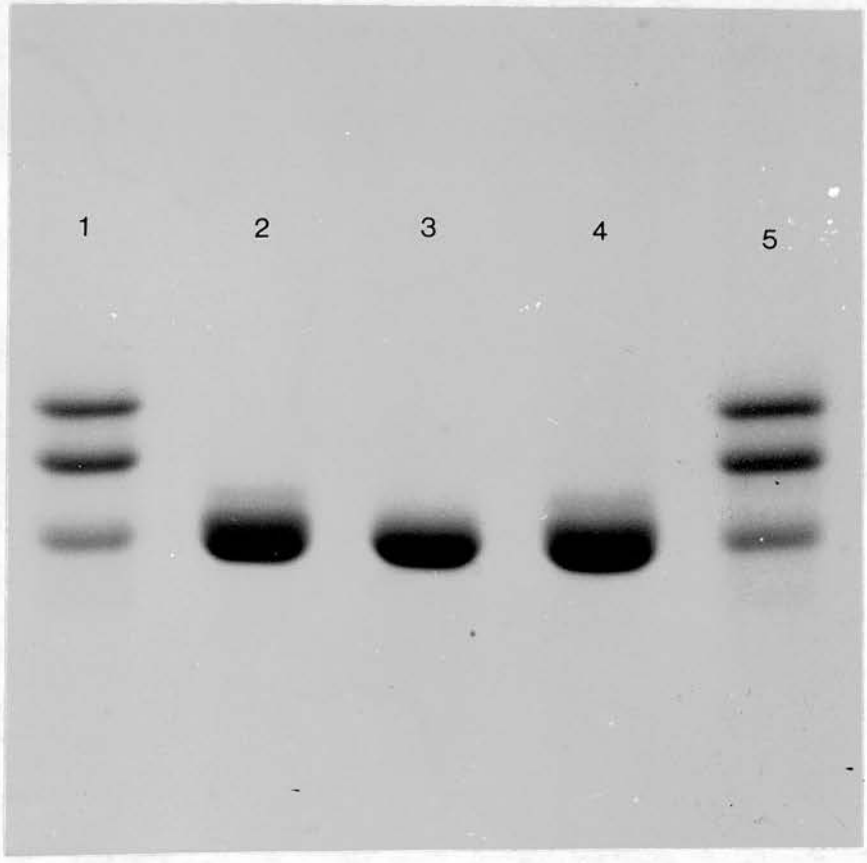

Figure 3e: Electrophoretic properties of human GST pi compared with a preparation from rat lung. The GST samples were analysed by SDS/PAGE as follows: 1, rat lung Yf (Mr 24 800), Yb (Mr 26 300) and Yc (Mr 27 500) subunits; 2, human placenta; 3, human lung; 4, human erythrocyte; 5,rat lung Yf, Yb and Yc subunits.

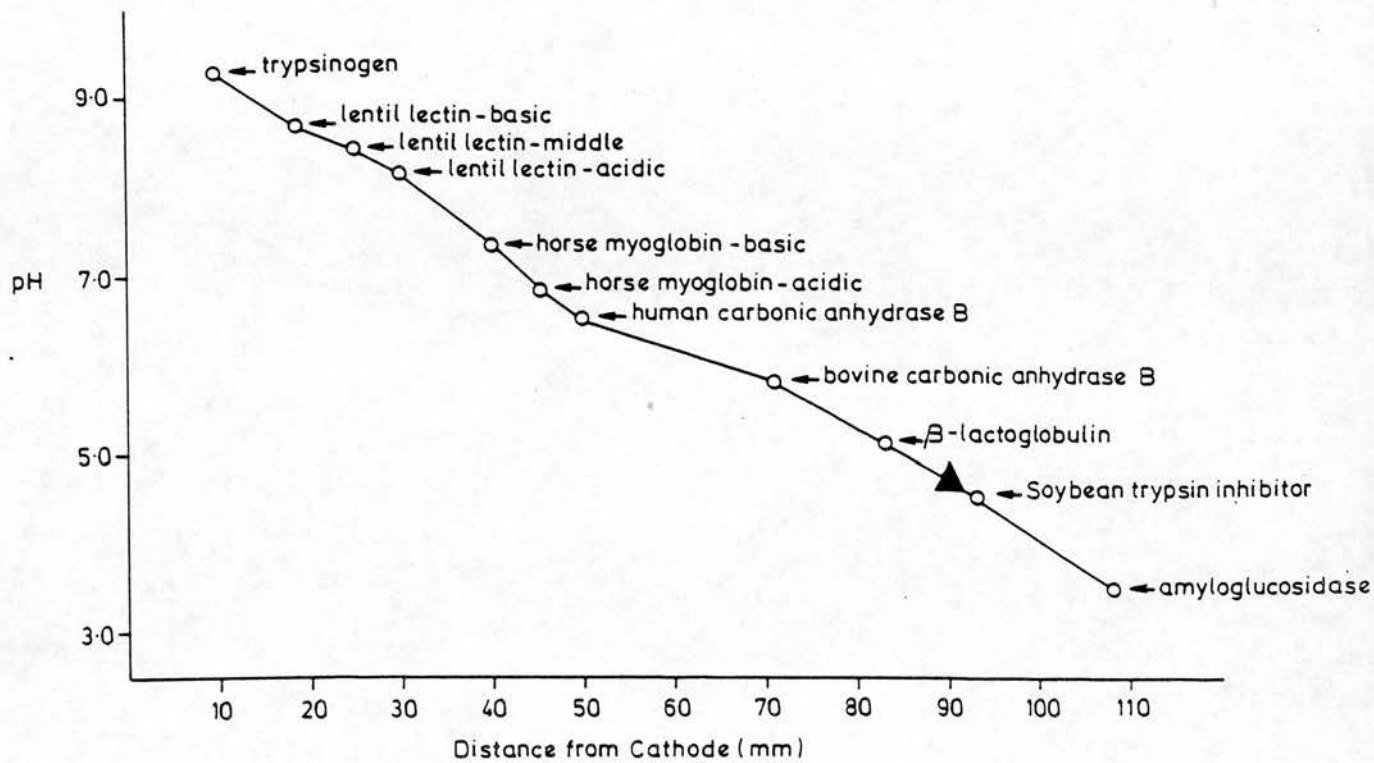

Figure 3f: Isoelectric focusing results. Illustrated are the relative distance each reference protein and GST migrated from the cathode. The distance the GST isoenzymes migrated is marked with a ▲.

3.06 : Discussion

The purification scheme developed for GST pi gave a high yield of homogeneous protein, and was found to be applicable to at least three different tissues that are rich in GST pi, placenta, lung and erythrocyte. The reason the glutathione-Sepharose 6B affinity gel was employed, as the final step, for erythrocytes was that initially the preparation was found to be contaminated with a slightly higher Mr weight polypeptide, if a S-hexylglutathione-Sepharose 6B affinity column was used. This band was thought not to be a GST, but possibly carbonic anhydrase, which has an acidic isoelectric point, a subunit Mr weight of about 29 000, known to be present in erythrocytes and reported as binding to S-hexylglutathione 6B affinity gel (Personal communication, B. Mannervik to J.D. Hayes).

The yield of GST pi obtained was about 5-fold greater than that reported for previous methods (Guthenberg *et al.,* 1979; Partridge *et al.,* 1984; Marcus *et al.,* 1978). Koskelo *et al.* (1981) described a purification scheme that resulted in the isolation of GST from human lung in amounts twice those reported here, but their preparations were only 90% pure when analysed by polyacrylamide gradient gel electrophoresis.

In this investigation, subunit molecular mass and the isoelectric point of the three GST enzymes isolated were found to be identical. Previous comparisons between the physicochemical and immunological properties of the pi class GST found in placenta, lung, erythrocyte, spleen and kidney has shown that these enzymes are closely related (Koskelo, 1983). However, using agarose gel electrophoresis (Suzuki *et al.,* 1987), GST pi from erythrocytes was found to have a mobility which differs from GST pi purified from other tissues. Similarly, during SDS/PAGE, Vander Jagt *et al.* (1985) showed that there was a molecular weight difference between placental (Mr 25 200) and erythrocyte (Mr 24 800) subunits of the GST pi. It has been suggested that these differences may be due to tissue-specific post-translational modification (Suzuki *et al.,* 1987). Together with the immunological data presented in Section 4 this data supports the widely held view that GST pi from placenta, lung and erythrocytes are genetically identical.

Section 4 : DEVELOPMENT OF A GST pi RADIOIMMUNOASSAY AND PLASMA MEASUREMENTS

4.01 : Preparation of antisera

Antisera were raised against human GST pi purified from lung and placenta. Purified GST (0.1 mg/mL) was emulsified with an equal volume of Freund's complete adjuvant. Freund's adjuvant (5 mL) was placed in a 25 mL universal on ice and an equal amount of protein solution, added drop-wise, with thorough mixing using a polytron homogeniser (Kinematica, Switzerland). The protein-containing emulsions were injected subcutaneously, at multiple sites, into female New Zealand White Rabbits (2 mL) , so that each rabbit received 100 μg of purified GST. Four rabbits were immunised with GST pi obtained from placenta and 4 rabbits with lung GST pi. After 6 weeks, each rabbit received a booster injection of immunogen (100 μg) prepared as described above in Freund's incomplete adjuvant. After a further two weeks, the animals were anaesthetized and blood recovered by cardiac puncture. The blood was allowed to clot before centrifugation at 3000 x g for 30 min, the serum removed and stored at -20 °C.

4.02 : Preparation of ^{125}I GST pi

Iodination of purified GST was performed as described in section 2.14. For iodination it was important to use purified GST pi that had been stored at -70 °C for less than 6 months. Purified GST pi that had been stored for longer than 6 months required re-purification as follows: Purified GST pi (2 mg/5 mL) from human placenta, in 20 mmol/L Tris/HCl buffer (pH 7.4 at 21 °C) containing 0.015% mercaptoethanol, was applied at 0.5 mL/min to a Mono Q, Fast Protein Liquid Chromatography (FPLC) column previously equilibrated with the same buffer. The column was developed with a gradient of 0-150 mmol/L NaCl in the equilibration buffer. Fractions (0.5 mL) were analysed for GST activity, protein concentration and sodium concentration. The appropriate fractions were pooled and dialysed (24 h at 4 °C) against 2 x 1 L, 50 mmol/L sodium phosphate buffer pH 7.4 and stored at -80 °C.

Figure 4a. shows the elution pattern obtained from the FPLC anion-exchange column. The second peak that eluted was used for iodination. Glutathione S-transferase activity using CDNB as substrate co-eluted with both protein peaks. Purified GST pi from the same preparation of human placenta (stored at -80 °C) has

been subject to re-purification on anion-exchange FPLC on three occasions, each re-purification yielding similar elution profiles.

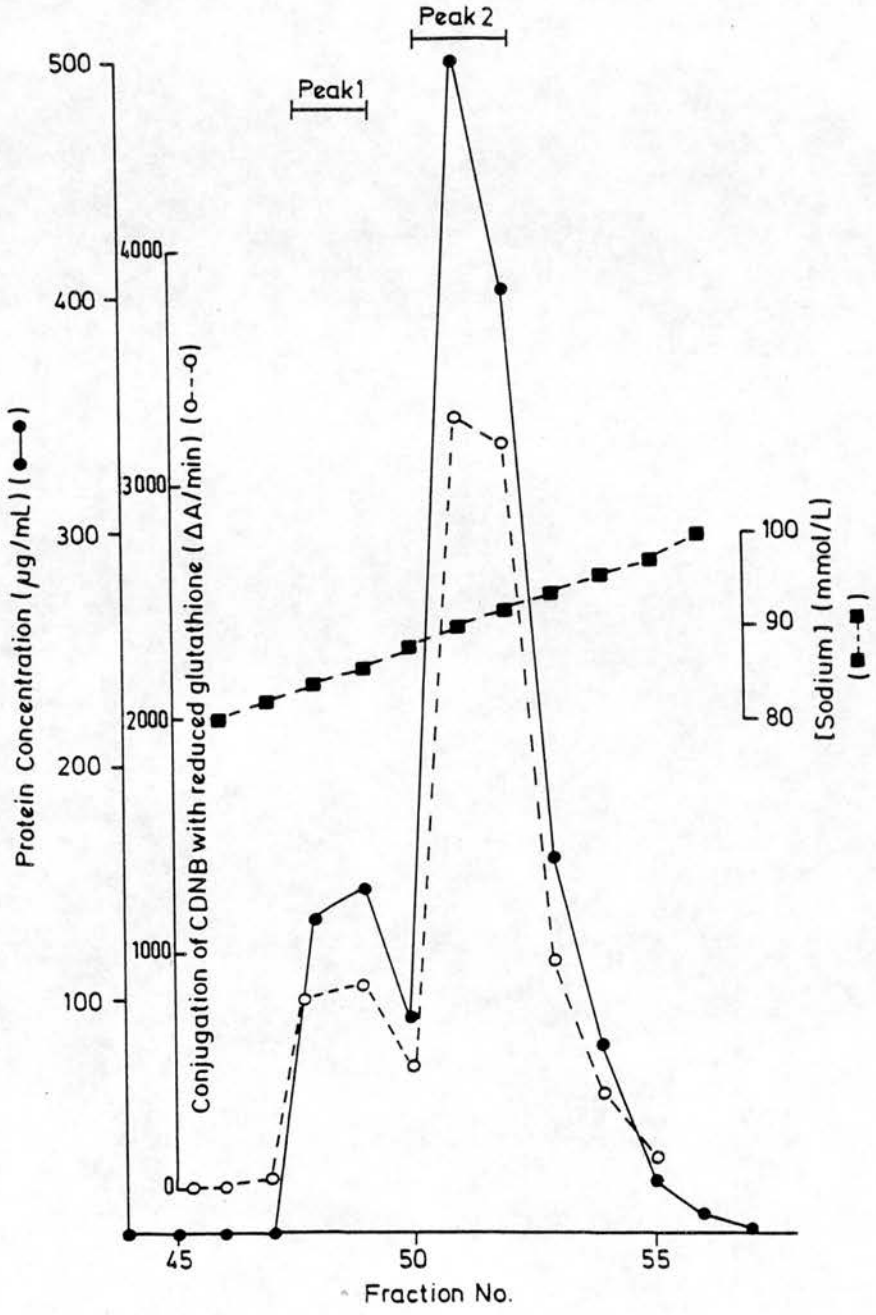

Figure 4a. Mono Q anion exchange column profile of GST pi. Protein concentration (●) was measured using the method of Bradford (1976). GST activity (O) was measured using CDNB as substrate.

Figure 4b illustrates a typical sephadex G-25 column profile obtained after the iodination of GST pi. The tracer in the first peak bound strongly to the GST pi antisera whereas, the second peak eluted in the salt volume was the free iodide peak and showed no binding to antisera. In this particular example, it was calculated that 33% incorporation of ^{125}I was achieved, and that the specific activity of the labelled protein was 1.2 MBq/μg. Fraction numbers 4 and 5, (estimated as containing 2.8 μg of labelled protein) were combined and stored at 4 °C until required. Iodinated protein was used within six weeks of preparation, thereafter precision became unsatisfactory due to decreased binding in the presence of no antigen, resulting in a loss of slope on the standard curve.

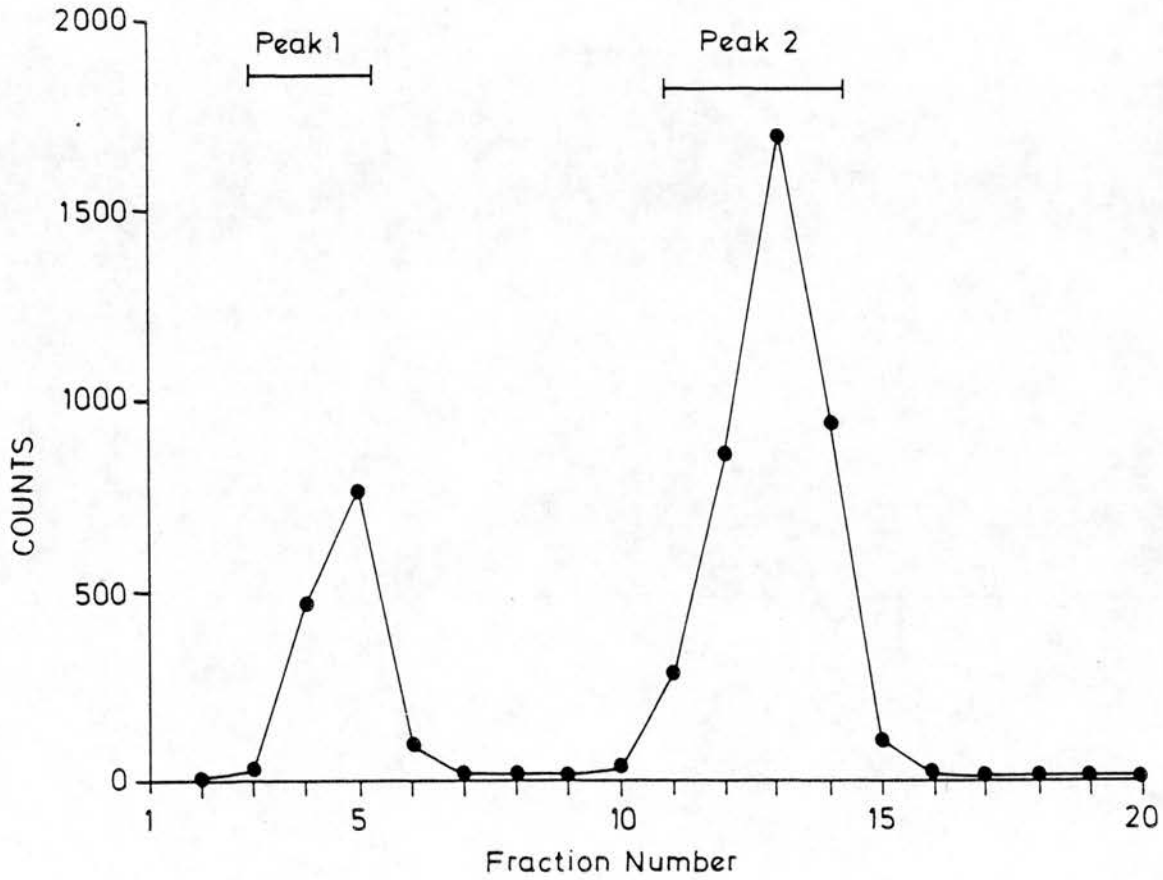

Figure 4b. Elution profile of iodinated GST pi after purification using Sephadex G-25.

4.03 : Assessment of antibody titre

A constant amount of the labelled antigen, ^{125}I-GST pi (100 μL: 40 000 cpm) was incubated with doubling dilutions of each antiserum (100 μL) and 100 μL of assay diluent, in triplicate and incubated for 24 h at 4 °C. The distribution of radioactivity bound to antibody and free fractions was determined after separation of bound and free fractions using pre-precipitated donkey anti-rabbit serum (DARS) (Section 2.14). Pre-precipitated DARS (100 μL) was added to each tube and incubated with shaking, at 21 °C for 1 h. A wash solution (2 mL; 0.05% aqueous Brij 35) was added to all tubes except the total counts, and the tubes centrifuged for 30 min (4 °C, 3000 x g). The supernatant was decanted and the precipitate counted in a LKB multigamma counter. The tracer bound to antibody, for each dilution of antibody, was plotted and the titre expressed as the amount of antisera required to bind 50% of tracer. Figure 4c. illustrates the different antibody dilution curves obtained for the 8 sera under investigation. The two antisera with the highest titres (Nos. 7 & 8),(1 in 3 000 initial dilution) were investigated further.

In order to assess the avidity of antisera 7 & 8 that showed the best titre, duplicate antibody dilution curves were set up as above, but in one curve the assay diluent was replaced by 100 μL of 10 μg/L GST pi standard. The difference in binding in the presence of diluent or 10 μg/L GST pi at each dilution of antisera was calculated. Figures 4d i & ii. illustrates the antiserum dilution curves for antisera numbers 7 & 8 with and without GST pi. As can be seen from the figures both antisera demonstrated equal separation between the two curves, with maximal differences in binding occurring at an initial antibody dilution of 1 in 1000. Antibody titres for each antisera are shown in table 4a.

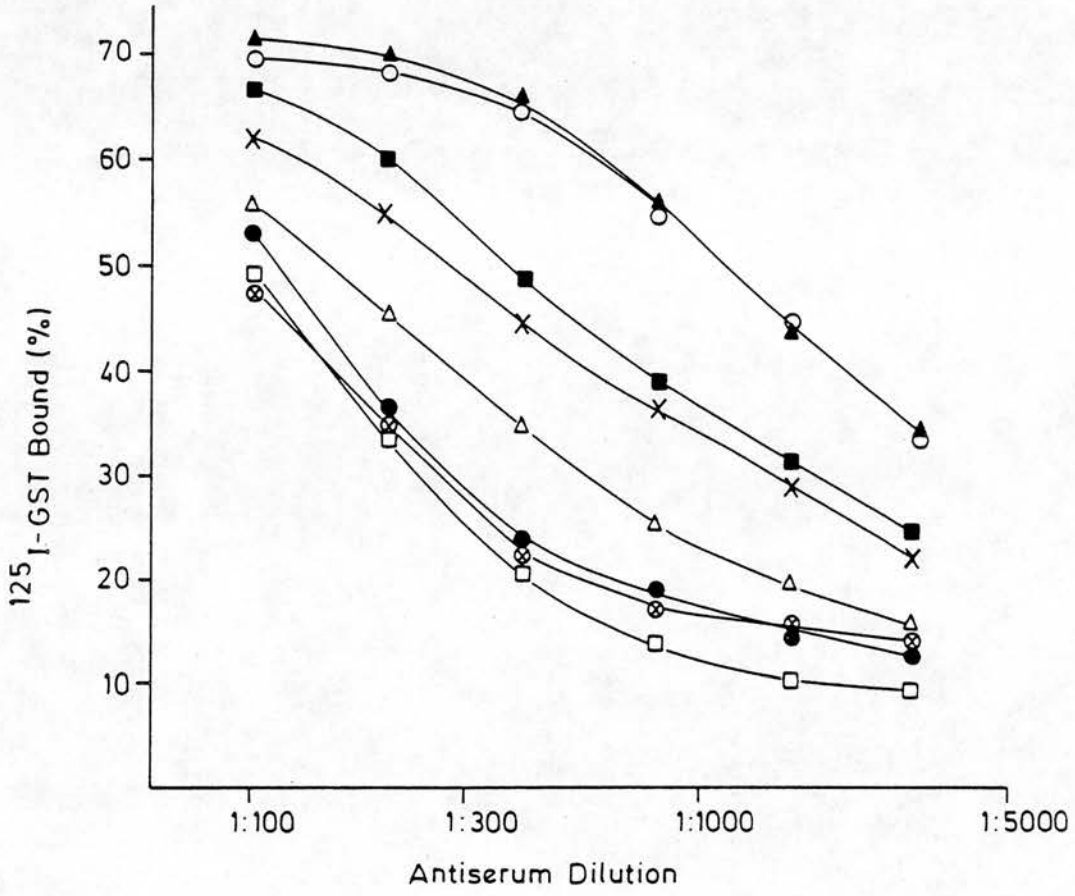

Figure 4c. Antiserum dilution curves using doubling dilutions of sera from 8 rabbits. Symbols ■,X,△,⊗,□,●,▲ and ○ correspond to rabbit numbers 1 to 8 respectively.

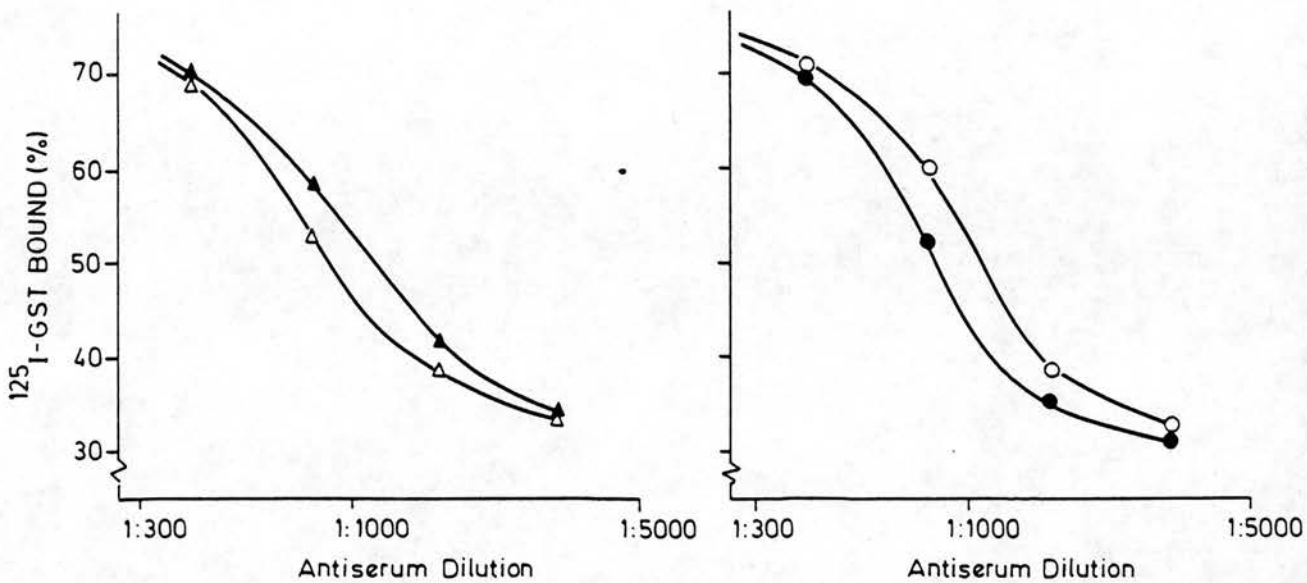

Figure 4d,i. Antisera dilution curves with (▲) and without (△) 10 μg/L GST pi for antiserum number 7.

Figure 4d,ii. Antisera dilution curves with (○) and without (●) 10 μg/L GST pi for antiserum number 8.

4.04 : Preparation and storage of GST pi standards

The protein concentration in the stock solution of GST pi was determined by the method of Bradford (1976) and working standards were prepared and stored at -20 °C in 0.5 mL portions. Initially, (1000, 500, 250, 100, 50, 25, 10, 5 μg/l) were prepared in assay diluent, which contained 1 g/L bovine serum albumin to reduce adsorption losses and stabilise the GST pi. Standards were prepared from GST pi purified from either placenta, lung and erythrocytes.

Latterly, when it became apparent that heat treatment of serum could destroy GST pi immuno-activity, pooled serum, obtained from blood donors, was heated at 55 °C for 30 min and used in the preparation of GST pi standards.

Heat treatment of serum: Purified GST pi from placenta was added to pooled human serum (obtained from blood donors) to give a total concentration of 125 μg/L. Portions were then heated at 25, 31, 36, 41, 49 and 55 °C in a water bath for 30 min, cooled and stored at -20 °C until the GST pi concentration could be measured by RIA. Figure 4e illustrates the GST pi concentration, as assessed by RIA, in serum that had been heated for 30 min at various temperatures. On the basis of this result, pooled serum was heated at 55 °C for 30 min and used in the preparation of GST pi standards for RIA.

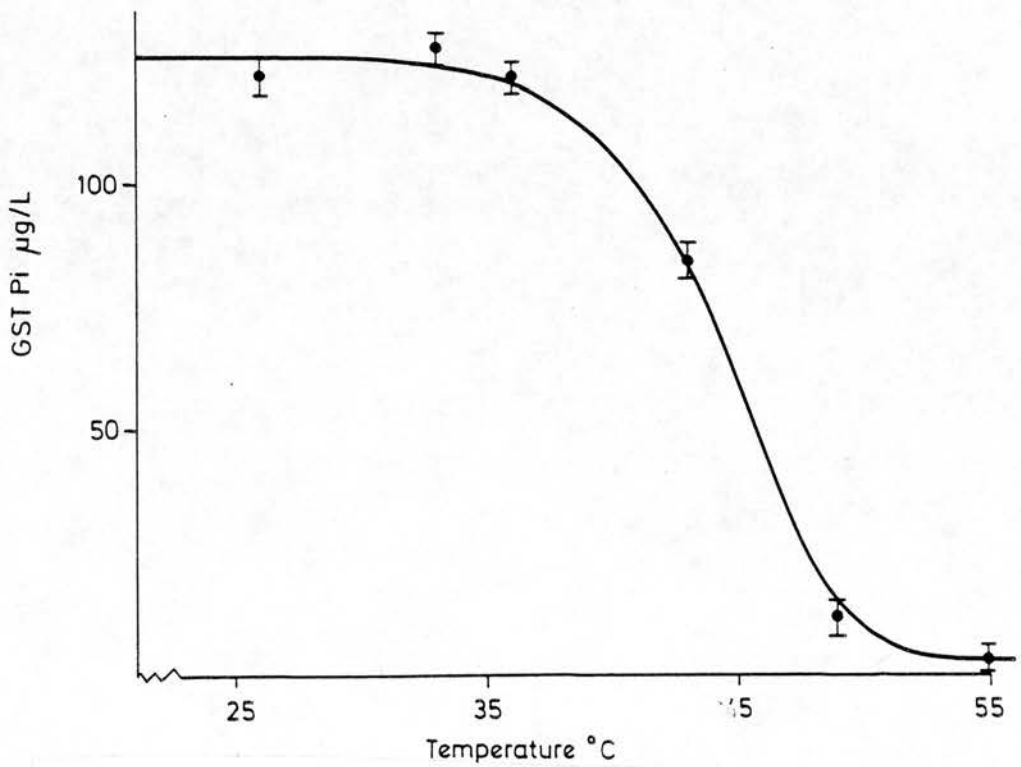

Figure 4e. GST pi (mean ± SD) concentrations in sera heated at various temperatures for 30 min.

4.05 : Assessment of antibody specificity

Radioimmunoassay is based on competition between the antigenic determinants of the labelled and unlabelled antigen in the presence of a limited number of specific antibody binding sites. Other substances that have similar structures to the antigen will also compete with the labelled and unlabelled antigen for the antibody binding sites and therefore cross-react with the antisera.

The cross-reactivity of three GST pi preparations obtained from placenta, lung or erythrocytes were determined as follows. Standard curves were set up in duplicate for each antigen. This was done for each of the 8 antisera under investigation at titres established from the antibody dilution curves. The labelled antigen in all cases was GST pi purified from placenta.

Figure 4f illustrates the example of antiserum number 8, when it was tested against the GST pi standards purified from the three different tissues, placenta, lung and erythrocyte. No significant difference could be seen in any of the dose response curves. This was also the result with the other 7 antisera tested.

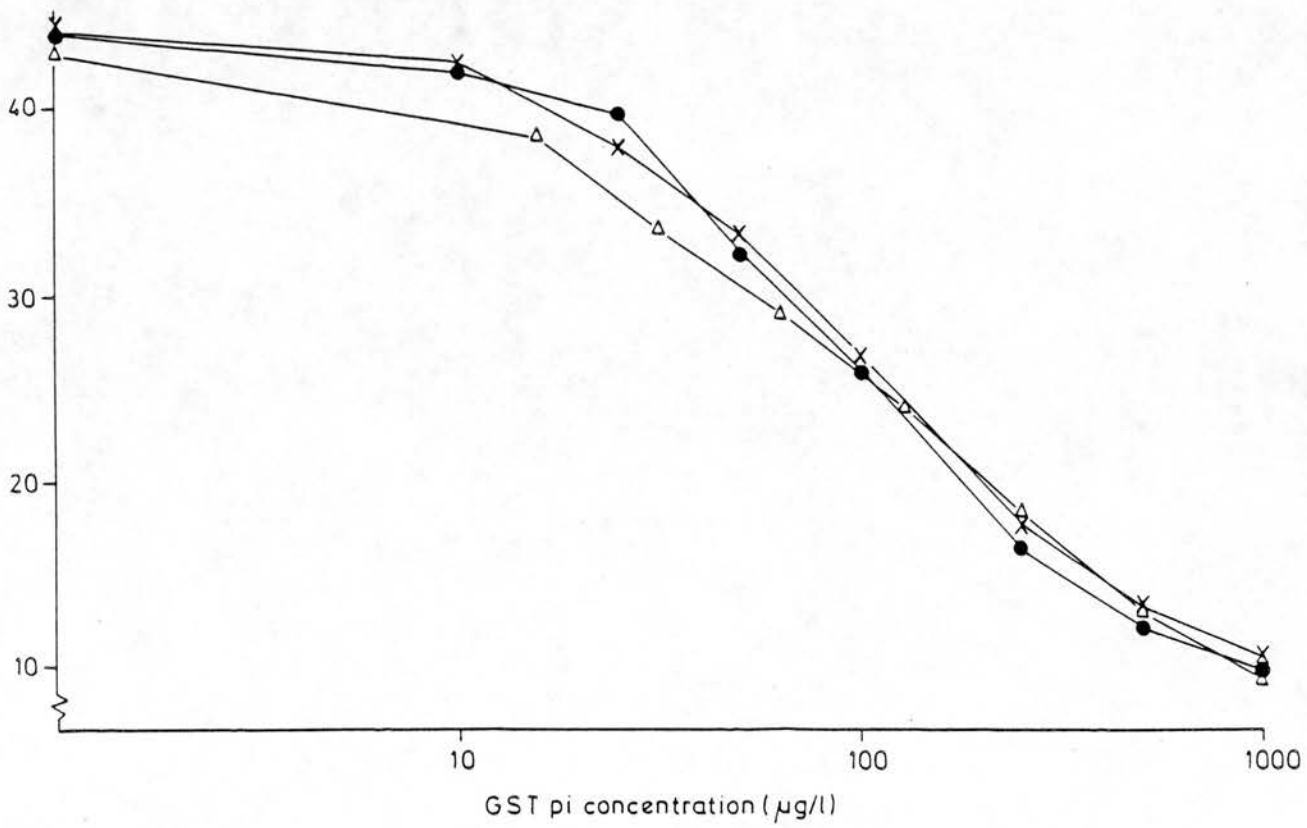

Figure 4f. A comparison of the dose response curves, with antiserum No. 8, against GST pi standards purified from placenta (●), lung (X) and erythrocyte (△). In all experiments ^{125}I-GST pi was placental in origin.

Cross-reactivity was assessed using the method of Abraham (1969) for human B_1, B_2, μ, ψ, rat Yf and mouse Yf GST for antisera numbers 7 and 8. In each case the maximum GST concentration assayed was 2 mg/L, again labelled placental GST pi was employed. Table 4a. illustrates the various titres and cross-reactivity characteristics of the various antibodies produced.

Table 4a: Titres and cross-reactivity of antisera raised to GST pi. Antisera titres are defined as the final dilution of antisera required to bind 50% of ^{125}I-labelled GST pi. The relative cross-reactivities are defined as the quantity of protein which was required to produce 50% displacement of bound ligand when compared with the original immunogen.

Antiserum	1	2	3	4	5	6	7	8
Immunogen	Lung	Lung	Lung	Lung	Placenta	Placenta	Placenta	Placenta
Titre (initial)	1:300	1:350	1:600	1:1200	1:350	1:1200	1:3000	1:3000
GST	**RELATIVE CROSS-REACTIVITY (%)**							
GST pi (placenta)	100	100	100	100	100	100	100	100
GST pi (lung)	100	100	100	100	100	100	100	100
GST pi (erythrocyte)	100	100	100	100	100	100	100	100
GST B_1	nd	nd	nd	nd	nd	nd	<0.1	<0.1
GST B_2	nd	nd	nd	nd	nd	nd	<0.1	<0.1
GST μ	nd	nd	nd	nd	nd	nd	<0.1	<0.1
GST ψ	nd	nd	nd	nd	nd	nd	<0.1	<0.1
RAT GST Yf	nd	nd	nd	nd	nd	nd	<0.5	<0.5
MOUSE GST Yf	nd	nd	nd	nd	nd	nd	<0.5	<0.5

nd, represents not determined.

4.06 : The effect of delayed tracer addition

The sensitivity of a RIA can be increased if addition of the tracer is delayed for 24 h after antigen and antibody have been incubated. This has been well described for RIA for GST B_1 and B_2 (Beckett and Hayes, 1984). Hussey *et al* (1987a) also employed this technique for increasing sensitivity in the RIA of GST μ. The use of delayed tracer addition was therefore investigated for the GST pi assay.

Two assays were set up in parallel, in one the antibody (100 μL), standard (100 μL) and tracer (^{125}I-GST pi: 100 μL) were added concurrently and the tubes incubated for 48 h before the separation of bound and free ^{125}I-GST pi by the addition of pre-precipitated DARS. In the other, antibody and standard were incubated for 24 h prior to the addition of tracer and a further incubation of 24 h before pre-precipitate DARS was added. Figure 4g illustrates the two standard curves obtained from the above experiment. The minimum detection limit, as defined by the lowest GST pi concentration giving a coefficient of variation (CV) of below 22.5%, was lowered from approximately 25 μg/L without delayed tracer addition to 7.5 μg/L with delayed tracer addition.

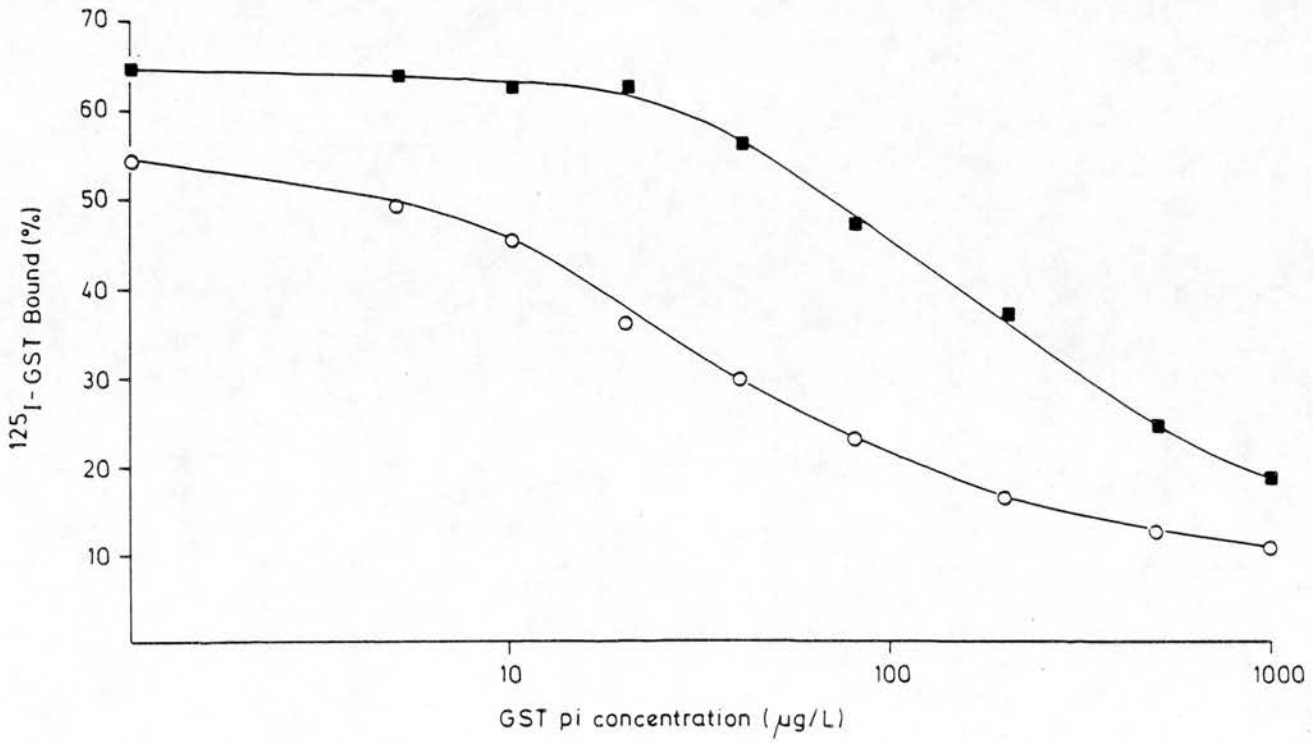

Figure 4g. The effect of delayed tracer addition on the calibration curves for the GST pi radioimmunoassay. Assay (●) used no delayed tracer addition whereas, assay (○) employed delayed tracer addition.

4.07 : GST pi radioimmunoassay protocol

The final protocol chosen for the GST pi RIA was as follows. Antibody 8 (raised from GST pi purified from placenta) was employed at an initial dilution of 1:1000. The antisera (100 μL) was incubated with 100 μL of standard or unknown for 24 h (4 °C) after which 100 μL (40 000 cpm) of tracer was added. After a further 24 h incubation (4 °C) 100 μL of pre-precipitate of DARS was added and the tubes shaken for 1 h at room temperature. The wash solution was then added (1.5 mL) and the tubes centrifuged for 30 min (4 °C, 3000 x g). The supernatant was carefully decanted and the radioactivity bound to the precipitate counted in a LKB 1261 Multigamma counter and the data processing performed using the LKB 1224-RIA Calc. LM evaluation program (LKB-Products, Bromma, Sweden).

4.08 : Precision of the GST pi radioimmunoassay

Three in-house plasma pools were prepared by collecting previously analysed samples and pooling groups of samples with similar GST pi levels. In order to obtain the high pool value a number of grossly haemolysed samples were included (approximately 20%). Pooled samples were mixed thoroughly, centrifuged at 3 000 x g for 30 min at 4 °C, then filtered through Whatman No. 1 filter paper before being portioned in 0.5 mL aliquots and stored at -20 °C, ready for analysis.

The mean within-assay precision, calculated from standard curves in 8 consecutive assays, is shown in figure 4h. The between-assay precision was determined using the in-house plasma pools. The precision was calculated from 17, 30 and 30 assays, for the low, medium and high pools respectively (table 4b).

Table 4b: Between-batch variation for 3 pools run in consecutive assays for GST pi.

	n	mean	SD	CV
POOL 1	17	31	2.9	9.4
POOL 2	30	70	6.6	9.5
POOL 3	30	250	11.9	4.8

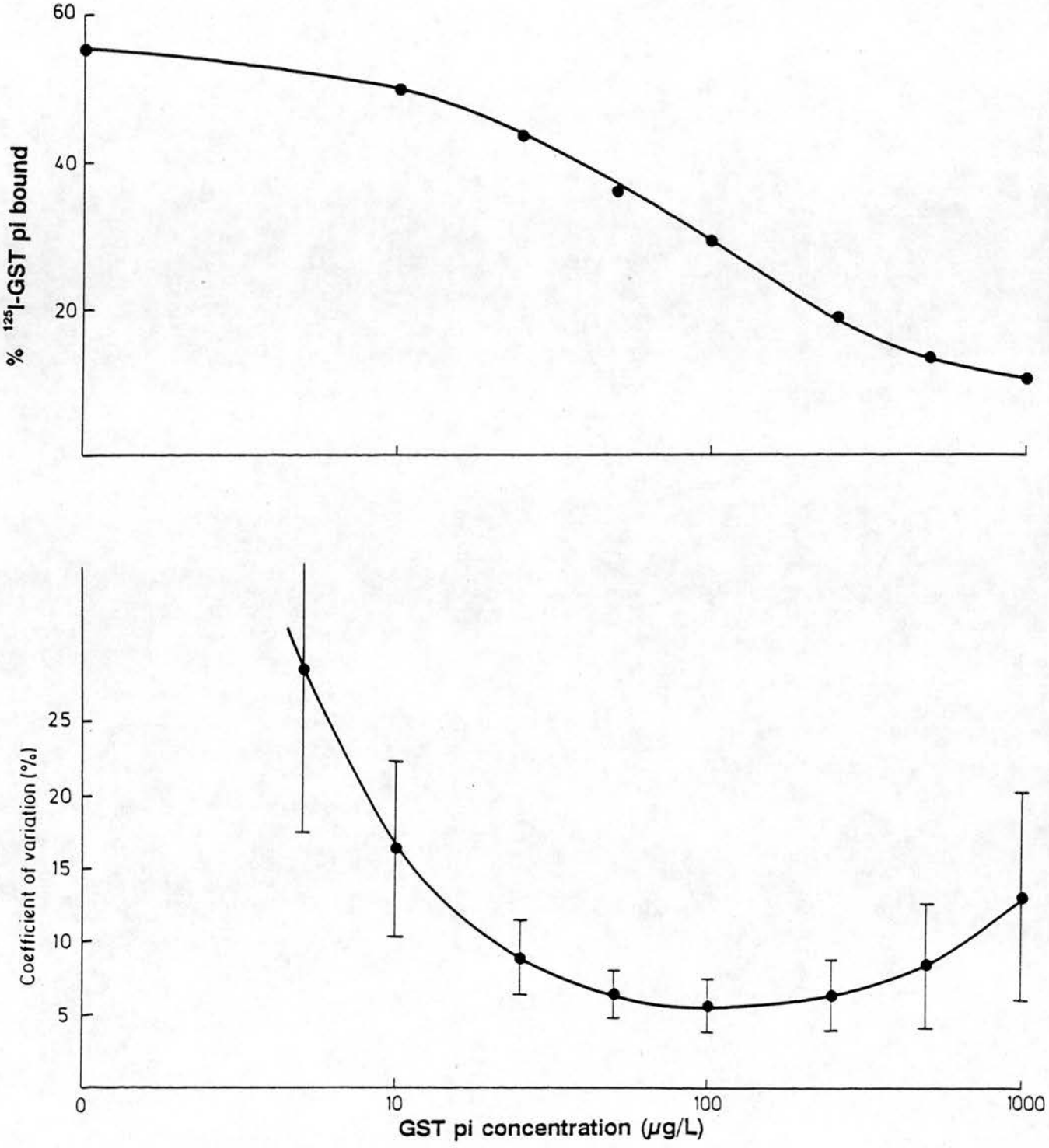

Figure 4h: An example of a standard curve and the mean inter-assay precision profile (± SD) for 8 consecutive human GST pi assays.

4.09 : Precautions required for the collection of Patient Plasma

Effect of haemolysis on GST pi concentrations in plasma: Bloods (n=14) were obtained from the Department of Clinical Chemistry's reception area, where they had been sent for routine biochemical analysis. The blood, collected into a lithium heparin tube, was divided into two portions, one was centrifuged at 3000 x g immediately for 15 min and the plasma stored at -20 °C until analysis, the other was stored at -20 °C without centrifugation and only centrifuged (3000 x g for 15 min) before analysis. The freezing and thawing of the whole blood caused gross haemolysis. The mean ± SD for the clear plasma was 43 ± 17 μg/L and the haemolysed sample 550 ± 405 μg/L.

Effect of delay in centrifuging blood samples on plasma levels of GST pi: In order to investigate the effects of any delay in separation of cells from plasma blood (40 mL) was collected without venous stasis from eight healthy volunteers into heparinised tubes. The blood was portioned and left either at 4 °C or room temperature for 0, 1, 2, 4, 6, 8, 24 and 48 h before being centrifuged at 3000 x g for 30 min at room temperature. The plasma was removed immediately after centrifugation and stored at -20 °C until analysis. Plasma samples showing signs of haemolysis were not assayed. The GST pi concentrations were measured by the RIA and the mean (± SD) results are shown in figure 4i. There was a significant ($P<0.01$; Student's *t*-test) increase in GST pi after 1 h; this was much larger for specimens kept at room temperature than at 4°C. After 1 h at room temperature there was no further significant increase in GST pi over the next 24 h. Although at 4 °C no significant rise was recorded over the first hour a significant increase ($P<0.05$) was recorded within 2 h. This rise in GST pi concentration was not as marked as that seen at room temperature (figure 4i.) although after 24 h, in both blood stored at room temperature and 4 °C, GST pi concentrations had increased 5 to 6-fold from its initial level. Further significant increases in GST pi concentrations were seen in the next 24 h at either temperature.

The initial (within the first 24 h) rise was thought be caused by the release of platelet GST pi and is discussed further. The secondary rise occurring from 24 h is probably due to leakage of GST pi from the erythrocytes.

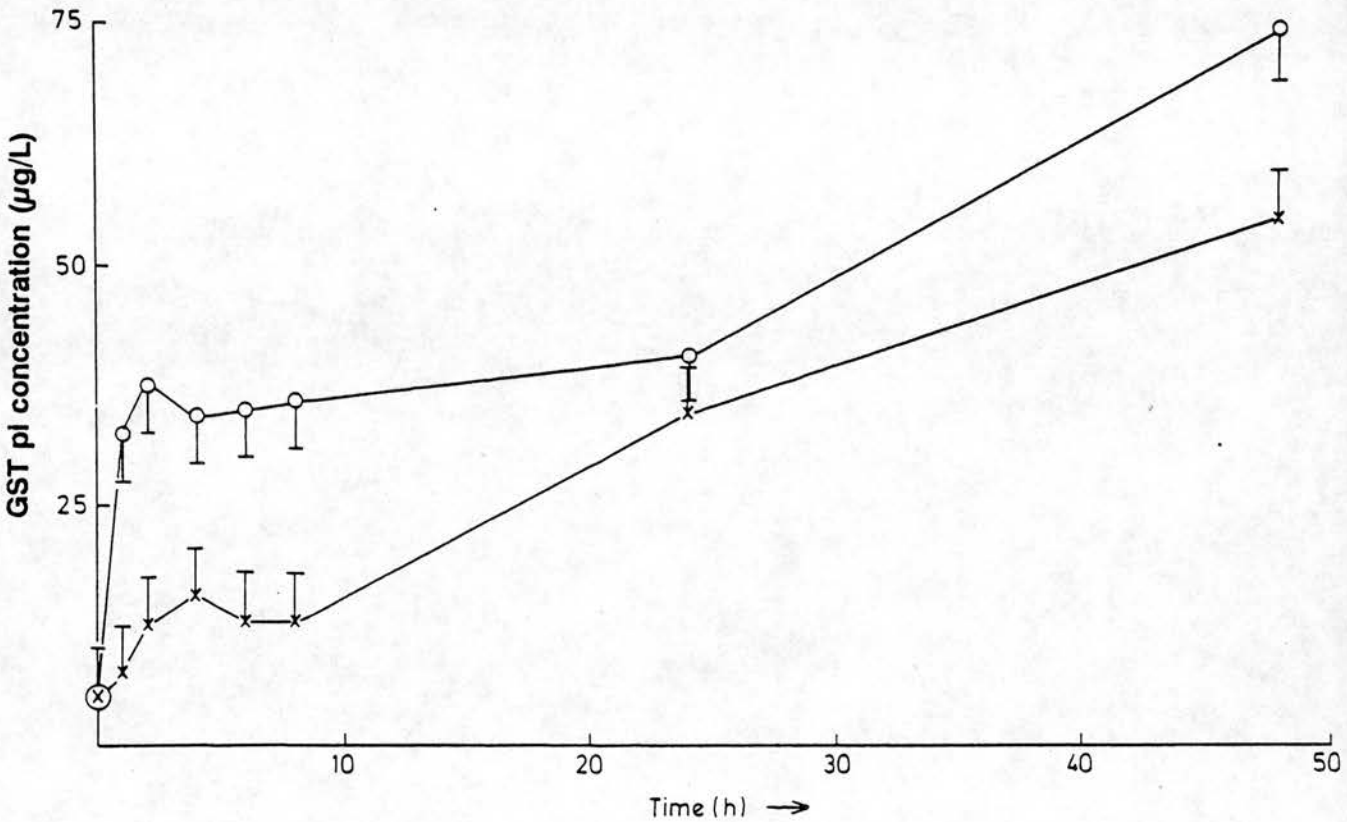

Figure 4i: Effect of delayed centrifugation of blood samples on plasma levels of GST pi. Samples stored at 4 °C before centrifugation (X). Samples stored at room temperature before centrifugation (O).

Effect of centrifugal force on plasma GST pi levels: Blood (30 mL) was taken without venous stasis from four healthy volunteers into heparinised tubes. Each specimen was divided into portions and centrifuged within 2 min at 150, 260, 580, 1040 and 2300 x g for 30 min at room temperature. Following centrifugation the plasma was separately immediately and stored at -20 °C until analysis. The mean (± SD) plasma GST pi levels in the plasma (figure 4j) show that a centrifugal force greater than 2000 x g is required to prevent plasma GST pi levels being elevated as a result of insufficient centrifugal force.

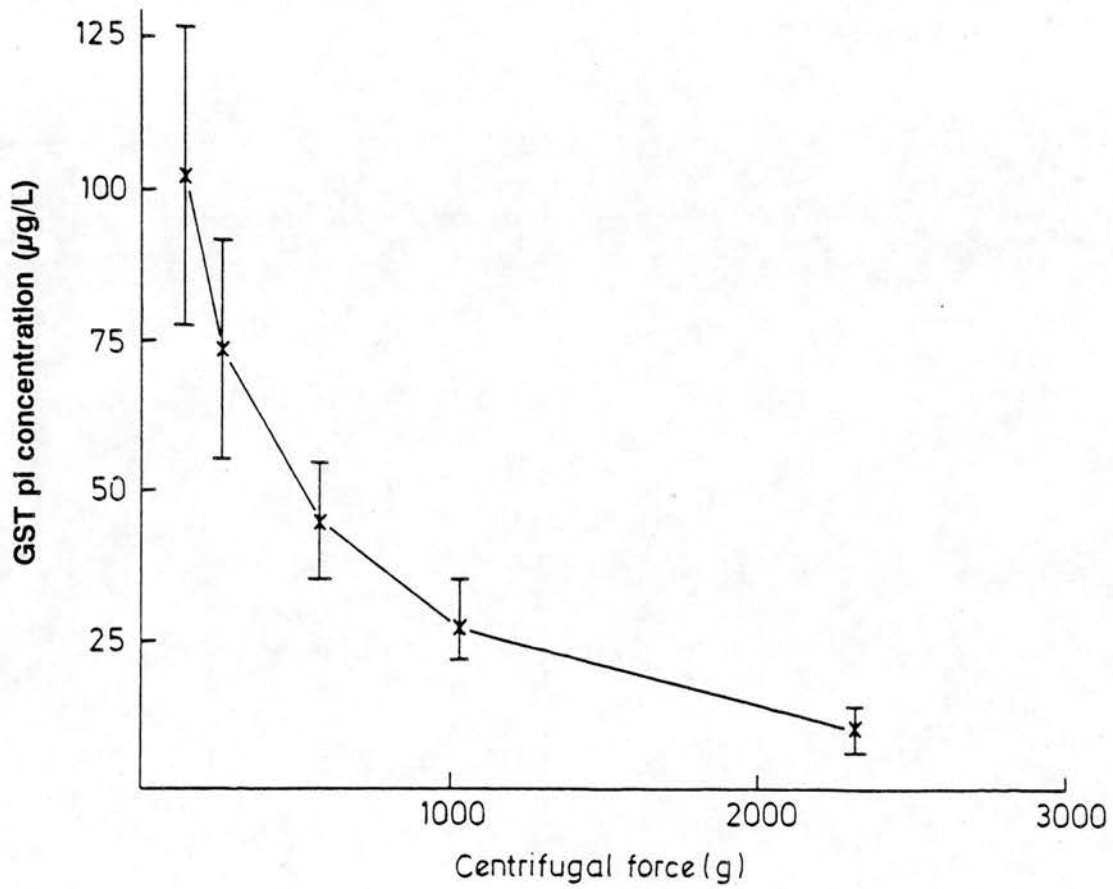

Figure 4j: Effect of centrifugal force on plasma GST pi levels.

4.10 : GST pi levels in platelet-poor and platelet-rich plasma

Samples of blood (18 mL) were taken without venous stasis from 12 healthy volunteers and added to 2 mL of a solution containing citric acid (8 g/L), trisodium citrate (22 g/L) and glucose (20 g/L) to prevent clotting. The blood was then divided into two equal portions (A and B). This citrate anticoagulant was recommended for the collection of platelet-rich plasma (Ludlam and Cash, 1976). Portion A was immediately centrifuged at 120 x g for 10 min at room temperature and the upper portion of plasma collected. Portion B was centrifuged at 2500 x g immediately at room temperature for 30 min; only the middle portion of plasma was collected. The samples were stored at -20 °C before analysis. The mean concentration (± SD) of GST pi in platelet-rich plasma was 154 μg/L (± 20.2). In all the samples of platelet-poor plasma GST pi concentrations were below 10 μg/L.

4.11 : Levels of GST pi in normal serum

Blood (10 mL) was collected from eight healthy volunteers into plain glass tubes and left to clot (1 h) at room temperature before centrifugation at 3500 x g for 30 min at 4 °C. Serum was removed immediately and stored at -20°C until analysis. The mean GST pi level in 8 sera was 16 μg/L (range 8-30 μg/L).

4.12 : Radioimmunoassay of Platelet Factor Four

Platelet factor four (PF_4) was carried out, essentially, according to the manufacturers instructions (Abbott Laboratories, Maidenhead, Berks., UK). All test reagents and specimens were brought to room temperature before beginning the test. Tubes were labelled for the performance of the test as follows:

a) Tubes 1 & 2, Total Count Tubes, for the determination of total radioactivity.

b) Tubes 3 & 4, Nonspecific Binding (NSB).

c) Tubes 5 to 14, for the appropriate standards in duplicate.

d) Tubes 15, etc. for the unknown specimens in duplicate.

Standard or unknown (25 μl) was incubated with ^{125}I-PF_4 (125 μL) and antiserum (125 μL) for 2 h at room temperature. Following this incubation, 0.5 mL of ammonium sulphate solution (73% saturated) was added to all tubes (bar numbers 1 & 2) and allowed to stand for 15-20 min, before centrifugation (3000 x g, at 4 °C) for 30 min. The supernatant was carefully decanted and the precipitate counted in a LKB 1261

Multigamma gamma counter and the data processing performed using the LKB 1224-Ria Calc. LM RIA evaluation program (LKB-Products, Bromma, Sweden).

4.13 : Effect of delay in centrifuging samples, collected into 'Thrombotect' tubes, on GST pi and PF_4 levels

Blood (20 mL) was collected without venous stasis from six healthy volunteers into 'Thrombotect' blood collection tubes (Abbott Laboratories, Maidenhead, Berks., UK), containing ethylenediaminetetra-acetic acid, 2-chloroadenosine and procaine/HCl; these additives prevented coagulation and platelet activation. The blood was portioned and left at room temperature for 0, 2, 4, 5 and 6 h before centrifugation (3500 x g for 30 min at 4 °C). Plasma was removed and stored at -20 °C before analysis for GST pi and PF_4. Figure 4k illustrates the mean (± SE) levels for the analytes during the time course studied.

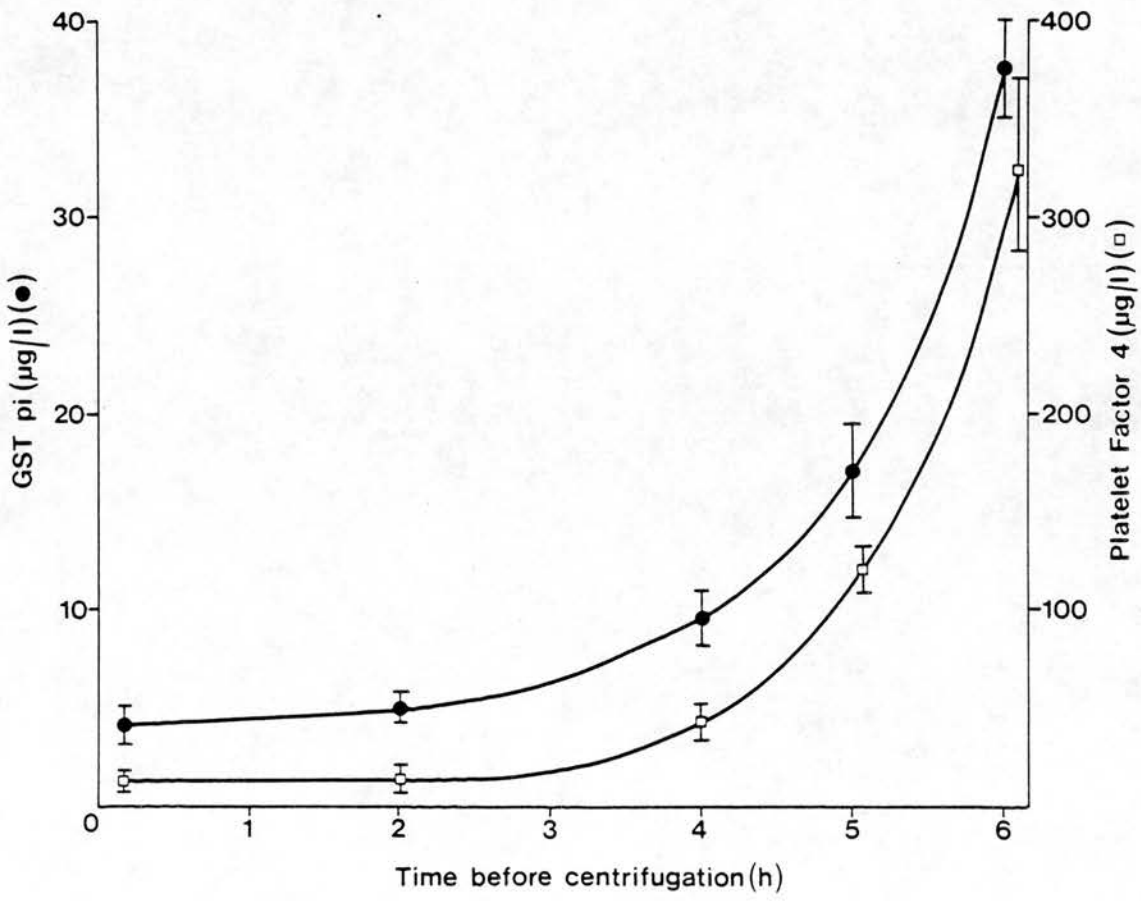

Figure 4k: Illustration of how GST pi and PF_4 concentrations increase with time, between sample collection and centrifugation.

4.14 : Investigation of plasma GST pi levels in patients with cancer of the bronchus

Control groups: Two control populations were studied, a group composed of healthy volunteers (n=26), and a group composed of patients with respiratory disorders other than cancer of the bronchus (n=16). The latter group consisted predominantly of sufferers from chronic obstructive airways disease and patients with pneumonia.
Lung cancer patients: Table 4c gives details of the 29 patients diagnosed as having carcinoma of the bronchus included in this study.

Blood samples were collected from atraumatic venepunctures with minimal venous occlusion and placed into 'thrombotect' blood collection tubes. The specimen tubes were labelled with the patient's name and the exact time the blood was taken. This labelling established whether the sample was received in the laboratory quickly enough for analysis (within 2 h). When received in the laboratory the specimens were immediately centrifuged for 30 min at 3000 x g (4 °C) and stored at -20 °C until analysis. In addition to measuring GST pi concentrations, PF4 levels were also measured. The assay of PF_4, an 8000 dalton protein that is secreted from the α-granules of blood platelets during the release reaction, was used to determine that appropriate sample collection and separation procedures had been followed and that no platelet activation had occurred. Any sample with a PF_4 concentration greater than 50 μg/L was excluded from the study. In practice, once the collection procedure had been established, only one sample with a high PF_4 concentration was encountered.

The GST pi level in all 26 healthy volunteers was less than 10 μg/L.
Figure 4l. illustrates the individual GST pi levels in the lung cancer patients (mean ± SE = 23 ± 7.2 μg/L) and the respiratory disease group (mean ± SE = 9 ± 0.7 μg/L). The median levels for the control group and the tumour group were 9 and 15 μg/L respectively. Utilizing a Mann-Whitney non-parametric test, the probability that the control group was different from the tumour group was $P<0.01$. The mean GST pi levels in the squamous cell carcinoma (n=11), adenocarcinoma (n=9) and small cell carcinoma (excluding patient 23) (n=5) were 12.3, 24.0 and 16.5, μg/L respectively. Eight out of 9 patients (89%) with adenocarcinoma had raised levels of plasma GST pi, whereas, only 3 out of 6 patients with small cell carcinomas and 6 out of 11 (but one was only 11 μg/L) of squamous carcinomas had elevated plasma GST pi concentrations. The one patient with large cell carcinoma had a plasma GST pi concentration that was within the reference range.

TABLE 4c: Patient characteristics and GST pi concentrations in the lung cancer group.

Patient	Sex	Age	Tumour Type	GST pi (µg/L)	PF_4 (µg/L)
1	M	60	Squamous	15	42
2	M	82	Squamous	8	50
3	M	81	Squamous	22	23
4	M	61	Squamous	7	49
5	F	43	Squamous	9	28
6	M	72	Squamous	21	15
7	F	52	Squamous	6	11
8	M	63	Squamous	11	<10
9	M	79	Squamous	5	<10
10	M	73	Squamous	21	50
11	M	67	Squamous	15	<10
12	M	76	Large Cell	8	<10
13	M	77	Adenocarcinoma	26	<10
14	F	67	Adenocarcinoma	14	<10
15	M	59	Adenocarcinoma	28	<10
16	F	66	Adenocarcinoma	18	25
17	M	63	Adenocarcinoma	33	<10
18	M	74	Adenocarcinoma	48	41
19	M	67	Adenocarcinoma	5	34
20	M	71	Adenocarcinoma	16	23
21	M	67	Adenocarcinoma	28	<10
22	M	53	Small Cell	10	<10
23	M	50	Small Cell	220	<10
24	F	65	Small Cell	6	<10
25	M	65	Small Cell	37	<10
26	F	68	Small Cell	13	<10
27	F	53	Small Cell	4	13
28	M	76	Histology inconclusive	16	38
29	M	78	Histology inconclusive	12	<10

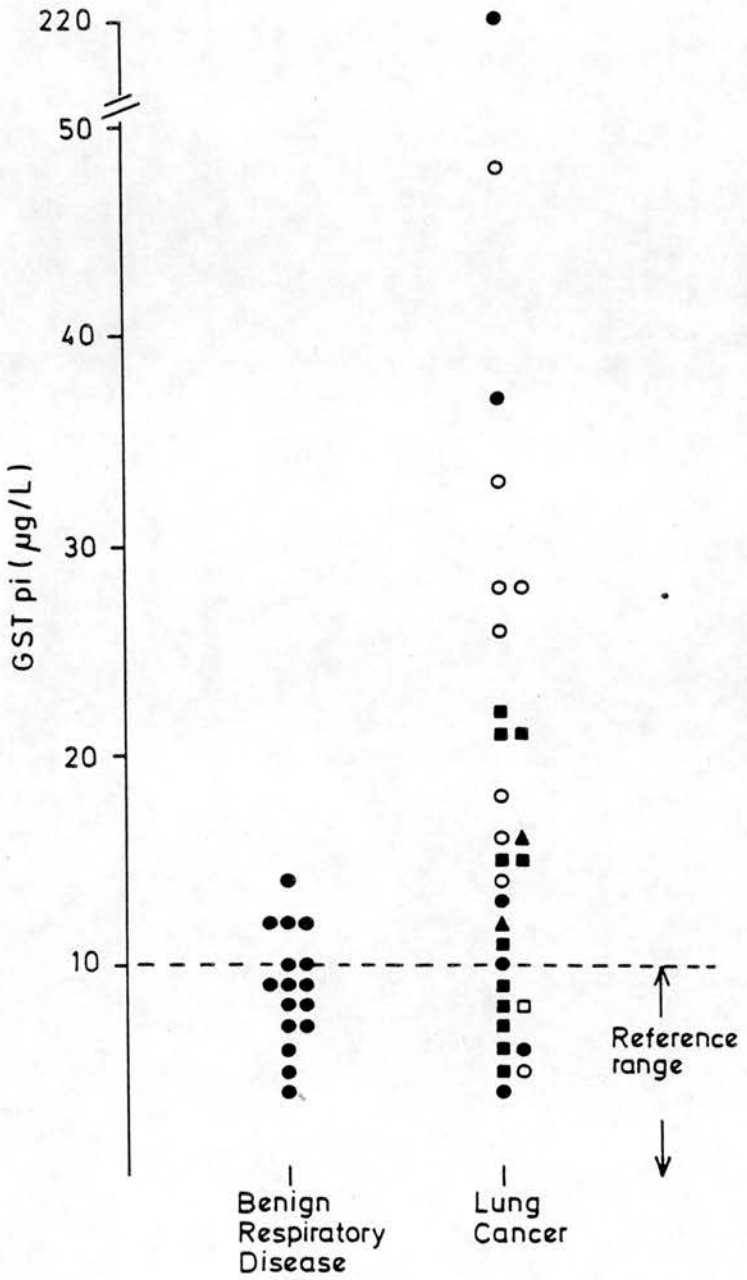

Figure 4I: GST pi concentrations in 16 patients with benign respiratory disease and 29 patients with carcinoma of the bronchus. Small cell, squamous, large cell and adenocarcinoma are represented by ●, ■, □ and ○ respectively. The 2 carcinomas of unknown type are represented by a ▲. Upper limit of reference range = 10 µg/L.

4.15 : Discussion

In this section, the development of a radioimmunoassay capable of measuring GST pi levels in plasma has been described. Antisera raised in eight separate rabbits to either lung or placental GST pi each showed 100% cross-reactivity with GST pi purified from placenta, lung or erythrocyte. This data coupled with the data from section 3 on the physicochemical properties of the enzyme, support the widely held view that GST π (placenta), λ (lung) and ρ (erythrocyte) are products of the same gene. The radioimmunoassay described here is specific for GST pi and demonstrates no cross-reactivity with the other human GST isoenzymes tested. The fact the antibodies did not cross-react with either the rat or mouse GST Yf is a demonstration of the specificity of the RIA technique. Kano *et al* (1987), looking at the structure and expression of the human GST pi mRNA and rat GST Yf mRNA deduced that both rat and human Yf subunits consist of 209 amino acids and they differ in only 30 amino acids (85.6% homology). Homology between different class GST (ie alpha, mu and pi), within the same species (rat), is only about 30% (Pickett *et al.*, 1984; Lai *et al.*,1986; Sugioka *et al.*, 1985).

In order to maintain optimal conditions for the RIA it was necessary to introduce an additional FPLC purification step for stored placental GST pi, because it became apparent that the purified GST pi, used for iodination, gave poor binding and precision when stored for more than 6 months. Chromatography of GST pi on the Mono Q anion-exchange column was employed as Radulovic and Kulkarni (1986) had reported separation of different interconvertible charge isomers of GST pi on a high performance liquid chromatography anion-exchange column. Whether the two peaks seen in figure 4a relate to the different charge isomers reported by Radulovic and Kulkarni (1986), or are simply the products of oxidation, is not known.

The data presented (figures 4h-j) illustrates the necessity for a strict sampling protocol if falsely elevated plasma GST pi levels are to be avoided. Delay in separation of plasma, or the use of an inadequate centrifugal force resulted in marked increases in the plasma concentration of GST pi probably as a result of the release of platelet GST pi into plasma. Federici *et al* (1985) and Loscalzo & Freedman (1986) have purified and characterised GST pi from human platelets. The 15-fold difference in GST pi which is measured between platelet-poor and platelet-rich plasma indicates that it is platelet GST pi which contributes to the marked elevation of GST pi plasma levels unless whole blood is centrifuged immediately after collection and with sufficient

centrifugal force. The introduction of 'Thrombotect' blood collection tubes inhibits platelet activation and hence the release of GST pi for up to 2 h (figure 4j), thus making collection of blood specimens more convenient and reliable. The assay of PF_4, an 8000 dalton protein that is secreted from the α-granules of blood platelets during the release reaction, was used to check that appropriate sample collection and separation procedures had been followed. From figure 4j it was concluded that significant release of GST pi from platelets did not occur until levels of PF_4 reached 100 μg/L, however an arbitrary cut of value of 50 μg/L for PF_4 concentrations was employed, samples with levels above 50 μg/L were excluded.

The value of serum but not plasma GST pi concentrations as tumour marker for gastrointestinal malignancies has been assessed (Niitsu *et al.*, 1989; Tsuchida *et al.*, 1989). Niitsu *et al.* observed elevated serum GST pi levels in patients with gastric, oesophageal, colonic, pancreatic, hepatocellular, and biliary tract cancers employing a immunoradiometric assay and suggested that it was a good tumour marker. Tsuchida *et al.*, employing an enzyme-linked immunosorbent assay, reported increased serum GST pi concentrations oesophageal cancer and pregnancy but not in lung cancer. Table 4c indicates, however, that the use of serum, rather than plasma obtained using 'Thrombotect' tubes, is inappropriate since platelet-derived GST would obscure any small increase in plasma GST pi produced by a tumour. Indeed, when GST pi was measured in serum from normal subjects, concentrations up to three times the upper level of the reference range from plasma were found. If an upper limit of 30 μg/L had been used for the reference range, which represents the highest value we measured in normal serum, only 4 out of the 29 cancer patients would have had elevated values for GST pi (Table 4c).

Using 'Thrombotect' tubes, a good discrimination between cancer and non-cancer patients could be made, with 19 cancer patients (66%) having elevated GST pi concentrations. It is perhaps significant that 8 out of 9 (89%) adenocarcinomas had elevated GST pi levels. A study (Rodenhuis *et al.*, 1987) has reported that out of 10 adenocarcinoma 5 had activated K-*ras* oncogenes; no *ras* gene mutations were observed in 15 squamous or 10 large cell carcinomas. In our study only 6 out of 11 of squamous carcinomas had modestly raised GST pi levels.

The GST pi gene contains an AP1 regulatory element 5' to the coding region (Cowell *et al.*, 1988) indicating that the induction of GST pi may be mediated through a *ras* responsive transcription. An inducible v-H-*ras* fusion gene, introduced into rat liver epithelial cells, has been shown to elevate the levels of GST pi (Burt *et al.*, 1988). The

greater plasma concentrations of GST pi in adenocarcinoma as compared to squamous carcinoma is also in agreement with mRNA levels in tumour tissue. In a comparison of normal versus tumour tissue, a 2.6 fold increase of GST pi mRNA in adenocarcinoma was observed compared to a 1.8 fold increase in squamous carcinoma (Moscow *et al.*, 1989).

Greater numbers of different classes of lung tumour types are required to evaluate fully the clinical usefulness of plasma GST pi measurements. Also the results of the tumour cytosol study described in section 6 and reports in the literature of increased GST pi levels in various tumour tissues warrant further investigation of plasma levels of GST pi. The radioimmunoassay for GST pi described here has sufficient precision, specificity and sensitivity to enable any changes that may occur in malignancy to be detected.

Section 5 : MEASUREMENT OF GST ISOENZYMES IN HUMAN BREAST CANCER CYTOSOLS

5.01 : Oestrogen receptors and breast cancer

Oestrogen receptors (ER) are specific oestrogen-binding proteins, termed oestrophilin, that are located in the cytoplasm and nucleus of oestrogen-responsive breast cells. Oestrogen receptors must be present for oestrogen to influence the biological activity and growth rate of breast tissue cells by increasing the production of mRNA and its subsequent translation into the corresponding protein. Approximately 50-70 % of female breast cancers have been found to contain oestrogen receptors. Although oestrophilins may exist in normal breast tissue to regulate breast tissue development during puberty and pregnancy, they are not usually present at measurable levels. The presence (ER-rich) or absence (ER-poor) of receptor protein and the concentration (estimated from the number of binding sites) in breast tumour tissue are established prognostic factors in the clinical course of breast cancer. Oestrogen receptor positive breast tumours are associated with improved response to hormonal therapy, longer disease-free intervals, and improved survival (McGuire *et al.*, 1975a&b; Nicolson *et al.*, 1981; Hawkins *et al.*, 1987; Courdi *et al.*, 1988).

5.02 : Association of ER status and GST pi

Using a cDNA probe, over-expression of GST pi mRNA has been found in a multi-drug resistant human breast cancer cell-line (Moscow *et al.*, 1988). The development of multidrug resistance in this adriamycin-resistant (Adr^R) MCF7 human breast cancer cell line is associated with a 45-fold increase in GST pi activity and with a concomitant loss of ER and hormonal sensitivity (Batist *et al.*, 1986; Cowan *et al.*, 1986). Moscow *et al.* (1988) also studied other breast cancer cell lines that were not selected for drug resistance, in each of these 5 additional cell lines an inverse relationship between GST pi activity and ER content was found. These same workers also examined the expression of GST pi mRNA in a small series of 21 breast cancers and again an inverse correlation between expression of GST pi and the ER levels in these tumours was reported.

Although GST pi mRNA is increased, it is not known if this is translated into a functional protein. Also the effects of ER status on the expression of the alpha or mu-class GST at the mRNA, DNA or protein levels have not been investigated. The studies reported in this chapter were performed to ascertain whether GST pi at the protein level has an inverse relationship with ER-poor tumours and to determine if GST

B_1, B_2 or μ have any association with ER status. The rate of GST μ expression in breast cancer was also determined to discover if, when compared to a disease free population, a greater or lower proportion of patients with breast cancer were nulled for this polymorphic GST isoenzyme.

5.03 : Breast tumour cytosol preparation

Cytosols were prepared from 58 breast tumours that had been stored in liquid nitrogen. All procedures were performed at 0 to 4 °C. Tumour tissue was dissected from surrounding fat and connective tissue, finely cut with scissors and homogenised in 20 mmol/L Tris buffer pH 7.5 (w/v 1:10) using a Silverson homogeniser at maximum speed on 20 sec, then 15 sec with 1 min intervals for cooling. The homogenate was then centrifuged at 105 000 x g for 1 h and the resultant supernatant was used as a cytosol.

5.04 : Measurement of oestrogen receptors

Oestrogen receptors were measured in an adjacent portion of tumour by saturation analysis (Hawkins *et al.*, 1981). Tumour cytosol was incubated overnight at 4 °C with [^{3}H] 17β-oestradiol. Separation of free and bound fraction was measured by liquid scintillation counting. Concentration of receptors was determined by Scatchard analysis (Scatchard, 1949). Activities below 20 fmol/mg cytosolic protein were designated ER-poor and those with activities in excess of 20 fmol/mg cytosolic protein as ER-rich.

5.05 : Measurement of GST concentrations in breast tumour cytosols

The specific radioimmunoassays for GST pi, B_1, B_2 and μ described in sections 2 and 4 were used to measure the GST isoenzymes. The cytosol, if required , was diluted in assay diluent consisting of 25 mmol/L sodium phosphate pH 7.6 bovine serum albumin (1 g/L) and sodium azide (0.2 g/L) before analysis.

5.06 : Protein measurement

The dye binding technique described by Bradford (1976) and adapted for use on a Cobas Fara centrifugal analyser ,as described in section 2, was used for total protein estimation.

5.07 : Results

Glutathione S-transferase pi, B_1 and B_2 were detected in all 58 breast tumour cytosols, GST μ was only detected in 28 (48%) of the cytosols. Table 5a summarizes the GST isoenzyme levels obtained.

Table 5a: GST levels in breast cancer cytosols. Results are expressed as μg GST/g protein. For GST μ, data is given for only the 28 tumours that express the protein (15 ER-rich & 13 ER-poor). The levels of GST pi were significantly higher in ER-poor tumours ($P<0.01$, Mann-Whitney U test) as compared to levels in ER-rich tumours.

	GST B_1		GST B_2		GST pi		GST μ	
	mean (±SD)	median	mean (±SD)	median	mean (±SD)	median	mean (±SD)	median
All tumours (n=58)	8.5 (±10.7)	4.5	1.2 (±1.1)	0.86	294 (±217)	222	174 (±174)	118
ER-rich (n=28)	5.2 (±4.6)	3.6	1.0 (±0.7)	0.98	208 (±76)	198	264 (±210)	183
ER-poor (n=30)	11.6 (±13.6)	4.7	1.4 (±1.3)	0.82	374 (±271)	306	160 (±122)	107

Glutathione S-transferase pi was expressed most strongly with concentrations ranging from 30 to 1110 μg/g. For the alpha-class GST B_1 and B_2 concentrations ranged from 0.6 to 48 μg/g cytosolic protein and 0.04 to 5.3 μg/g cytosolic protein respectively. Glutathione S-transferase μ concentrations, when expressed, ranged from 18 to 576 μg/g cytosolic protein. Indeed, GST μ was the most predominant GST isoenzyme present in 9 out of the 28 tumour cytosols that expressed the protein. A significant correlation, utilizing Pearson's correlation analysis ($r=0.66$, $P<0.001$), was observed between levels of GST B_1 and B_2. A summary of the other correlation coefficients are given in table 5b.

Table 5b: Summary of Pearson correlation coefficients between GST pi, B_1, B_2, μ and ER concentrations for the 58 breast tumours except for correlations with GST μ where only the 28 tumours expressing GST μ were used.

	GST pi	GST B_1	GST B_2	GST μ	ER
GST pi	1	0.37**	0.24***	-0.18	-0.24***
GST B_1		1	0.66*	-0.16	-0.26***
GST B_2			1	0.22	-0.18
GST μ				1	0.38***

*$P<0.001$, **$P<0.002$, ***$P<0.05$.

Concentrations of GST in the cytosols subdivided according to the ER status as shown in figure 5a. The levels of GST pi were significantly higher in ER-poor tumours ($P<0.01$, Mann-Whitney U test) as compared to levels in ER-rich tumours. The tumours with the 11 highest GST pi values (>400 μg GST/g cytosolic protein, the highest GST pi concentration seen in ER-rich tumours) were all in the ER-poor tumours. As with GST pi the highest levels of GST B_1 and B_2 were observed in the ER-poor tumours with the eight highest GST B_1 levels being found in the ER-poor tumour group. However when compared as groups the difference in the alpha-class GST between ER-rich and ER-poor tumours did not reach statistical significance. Minor correlations could be found in the quantitative levels of oestrogen receptors and the expression of the GST isoenzymes (table 5b). However, no correlation was found between ER level, in the ER-rich group, and each of the GST isoenzymes.

The frequency with which GST μ was expressed in the breast cancers (48%) was not significantly different from the frequency of expression of GST μ found using lymphocytes obtained from a group of 42 laboratory volunteers (described in section 7) where 23 (55%) volunteers expressed the enzyme (Chi square = 0.19; $P=0.66$). Also no association was found between GST μ and ER status, GST μ was present in 15 of the 30 ER-poor tumours and 13 of the 28 ER-rich tumours.

The same pattern of GST expression between groups was observed if a level of >5 fmol/mg was used to define ER status. In the literature the cut-off for defining whether a tissue is ER-rich or -poor varies from >5 to >30 fmol/mg. Three tumours would have been reclassified as ER-rich, had a cut-off of >5 fmol/mg been employed. All 3 samples had GST pi, B_1 and B_2 concentrations which overlapped whether they were classified in the ER-rich or -poor group. Two of the samples expressed GST μ, however this did not effect the expression rate of this isoenzyme, for either group significantly.

Although patient details were not available on all samples, there was no relationship between GST levels and the age or menopausal status of the patient from whom the sample had been derived. Likewise, no apparent association could be determined between GST levels and lymph node involvement or tumour T stage in the group of patients for whom this information was available. Similarly no special histological tumour type was associated with increased levels of GST.

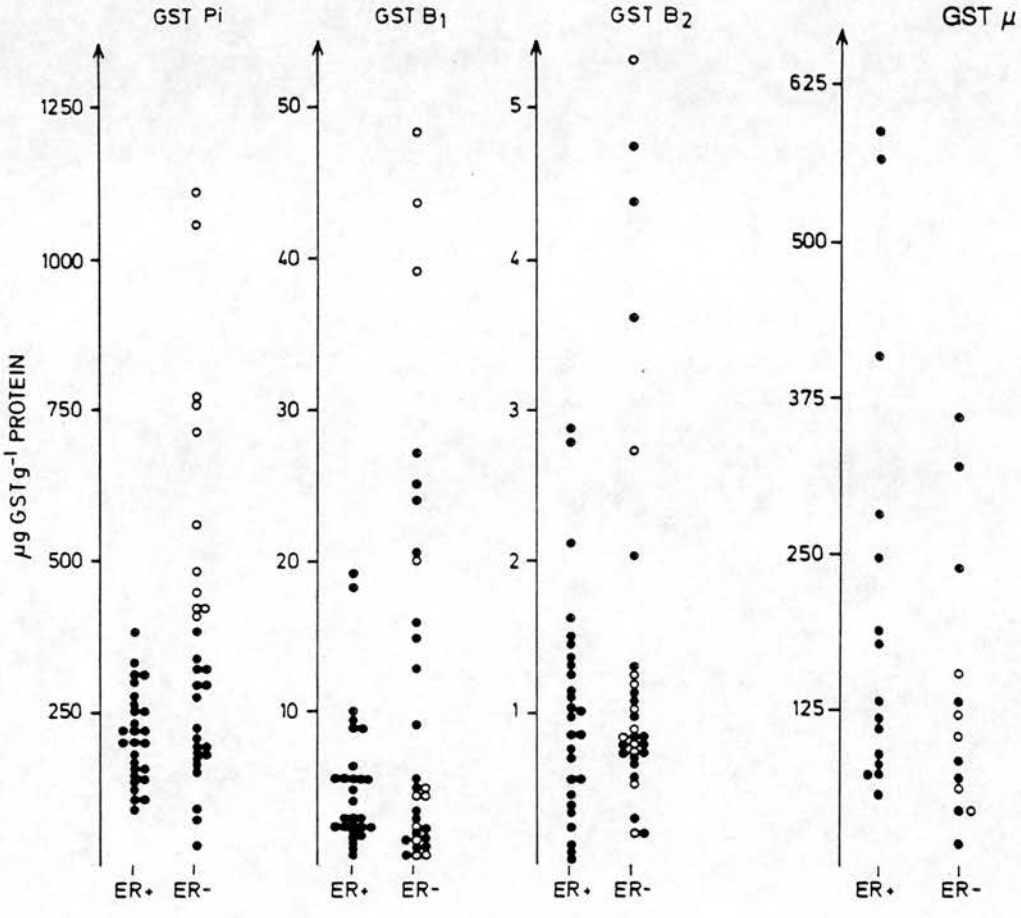

Figure 5a: Concentrations of GST B_1, B_2, pi and μ in oestrogen rich (ER+) and oestrogen poor (ER-) breast tumours (●). Tumours in which the concentration of GST pi exceeded 400 μg/g cytosolic protein are shown by (○).

5.08 : Discussion of GST levels in breast tumour cytosols

In this study, all three classes of GST isoenzymes were detected in breast cancer cytosols, whereas Di Ilio *et al.*, (1986) reported that only GST pi was present in normal or tumour breast cytosols. Although, GST pi was the major GST isoenzyme encountered in the majority of cytosols examined, GST μ when expressed had levels approximately 50 % that of GST pi. The levels of alpha-class GST recorded in this study were low (see section 6 for a comparison with other organs) and thus it is not surprising that Di Ilio *et al.*, (1986) did not detect alpha-class GST by GSH-affinity column and chromatofocusing chromatography. The observation that higher GST pi concentrations are found in ER-poor breast cancers as compared to ER-rich are in agreement with the data of Moscow *et al.* (1988), who showed increased mRNA levels for GST pi in a similar group of patients. The increased GST pi concentrations in the cytosol indicates that the mRNA is being translated. In addition, to the GST pi data, it has also been shown here, that the alpha-class GST appear to be expressed in greater concentrations in ER-poor as compared to ER-rich breast tumours.

ER-poor tumours have been associated with a worse prognosis than ER-rich tumours. Whether this observation is due to ER-poor tumours having a higher proliferation rate (Meyer *et al.*, 1977) of the two types of tumours or is simply due to differences in the sensitivity of these tumours to chemotherapeutic agents is not clear. The evidence that GST are associated with the phenomena of drug resistance is substantial (for a review see Hayes & Wolf, 1988). Therefore the finding of increased GST pi in ER-poor tumours may add weight to the theory that ER-poor tumours are more resistant to chemotherapy, however GST pi as yet, has not been implicated in the direct metabolism of any anticancer therapeutic agent.

The ligand-binding properties of GST rather than their detoxification properties may be important in cellular protection. The GST have been shown to bind steroid hormones including, corticosterone, testosterone, progesterone and 17β-oestradiol (for a review see Listowsky *et al.*, 1988). These binding experiments were performed by circular dichroism spectra of rat Yb2-GST-bilirubin complex to study the competitive displacement of bilirubin from the protein by other ligands (Kamisaka *et al.*, 1975). This same work, reports that the rat Yb2 GST has a weak affinity for tamoxifen the drug currently used in the hormonal deprivation therapy of breast tumours. Tamoxifen acts by irreversibly binding the ER thus blocking the any possible action of 17β-oestradiol on the nucleus. Rat Yb2 GST a mu-class GST, may have completely different binding characteristics than human Yf (GST pi) GST.

It should be emphasised that whereas the majority of ER-poor tumours rarely respond to hormone manipulation (Le Clercq & Heuson, 1977), in this study only a small proportion of ER-poor tumours had elevated levels of GST. In this respect the observation that ER-poor breast cancers can be further subdivided into groups on the basis of other tumour characteristics such as epidermal growth factor receptors, vimentin and p53 expression (Cattoretti *et al.*, 1988) is probably relevant. Unfortunately, I did not measure these parameters and therefore cannot conclude if there is an association between GST pi expression and epidermal growth factor receptor levels. It would have been of interest to know whether the patients with tumours expressing the higher levels of GST return more quickly with recurrent disease than those with lower GST, irrespective of ER status but follow up details were not available.

A study involving the assessment of GST expression in a series of 86 primary human breast cancers by an immunohistochemical method (Cairns *et al.*, 1989), concluded that their results did not support the hypothesis that local levels of GST determine response to chemotherapy. An association was observed between GST pi staining and histological grade and stromal elements (eg. presence of fibroblasts, inflammatory cells), no reference to ER status was made. The possibility that in this study, the elevated GST pi concentrations are primarily due to stromal effects is unlikely as with 58 samples one would have expected to see an equal distribution in ER-poor or ER-rich groups of stromal elements. The immunohistological technique only detected the alpha-, mu- and pi-class GST in 13, 51 and 43 % of the breast tumours respectively whereas I was able to detect the alpha- and pi-class GST in all samples. This difference is probably due to the different sensitivities of the two techniques and raises the question whether immunohistochemistry is a sensitive enough technique to look at the small differences in expression of GST shown in breast cancers.

Glutathione S-transferase pi maps to chromosome 11 (Moscow *et al.*, 1988) which has an increased incidence of deletions in ER-poor tumour cells when compared to ER-rich tumours (Ali *et al.*, 1987). These observations have led to the suggestion of a link between over-expression of GST pi and a specific deletion on chromosome 11. The alpha-class of GST, however, map to chromosome 6 (Board & Webb, 1987) and this data shows that both GST B_1 and GST B_2 are also over-expressed in a number of ER-poor tumours. This suggests that there may be a common mechanism rather than specific chromosomal deletions that leads to the elevation of GST concentrations in

certain breast cancers.

The incidence of GST μ expression in both the ER-poor and ER-rich breast tumours was not significantly different from the incidence of GST μ expression found in lymphocytes from the normal population. Heavy smokers are reported to be more susceptible to lung cancer if they lack GST μ (Seidegard *et al.*, 1986). The results of this study indicate there is no association between expression of GST μ and susceptibility to breast cancer.

Section 6 : GLUTATHIONE S-TRANSFERASE LEVELS AND GLUTATHIONE PEROXIDASE ACTIVITIES IN HUMAN TUMOURS

The central role of the glutathione S-transferases and glutathione peroxidase in chemical detoxification has led to the recognition that these proteins are probably involved in the sensitivity of tumours to the therapeutic effects of anticancer drugs (Hayes and Wolf, 1988). There is an increasing body of evidence to indicate that this is indeed the case. For example, cell lines made resistant to anticancer drugs over-express GST proteins (Batist *et al.,* 1986; Robson *et al.,* 1987; Lewis *et al.,* 1988; Wang and Tew, 1985). In one report this has been shown to be due to an amplification of an alpha-class GST (Lewis *et al.,* 1988). It has also been recently shown that the expression of human alpha- or pi-class cDNA in yeast confers resistance to the anticancer drugs chlorambucil and adriamycin (Black *et al.,* 1989). On the basis of this direct evidence, that these proteins will be a factor in chemotherapy, it is important to establish the levels of GST isoenzymes in human tumours.

There have been several reports on the expression of GST mRNA or protein in human tumours (Carmichael *et al.,* 1988; Di Ilio *et al.,* 1988b; Di Ilio *et al.,* 1987a; Eimoto *et al.,* 1988; Moscow *et al.,* 1989). However, most of these deal only with the GST pi isoenzyme. In order to examine further the GST expression in normal and tumour human tissue, in this study, the composition of glutathione S-transferase (GST) isoenzymes in human normal and tumorous lung, colon, stomach, breast, kidney and liver tissue have been examined by a variety of techniques. The levels of GST activity were assessed by measuring the rate of conjugation between 1-chloro-2,4-dinitrobenzene (CDNB) and glutathione. In addition, selenium-dependent and independent glutathione peroxidase activities were measured in the tissues using hydrogen peroxide (H_2O_2) and cumene hydroperoxide (CuOOH) as substrates, respectively. Individual subunit quantification was performed by the sensitive and specific technique of radioimmunoassay (RIA). Western blot analysis, probing with antisera from the three different classes of GST alpha, mu and pi, was employed to check for any novel polypeptides highlighted by these antisera.

6.01 : Matched normal and tumour cytosol preparation

Tumour and adjacent normal tissue from patients who had not received prior chemotherapy, were obtained at surgery. Tissues were analysed and dissected by surgical pathology. Matched tumour and normal tissue from lung (19), colon (27), stomach (9), breast (18), and kidney (7) was collected. The lung tumours comprised 7 adenocarcinomas, 6 squamous carcinomas, 3 large cell carcinomas, 1 carcinoid, 1 mesothelioma and a metastatic histiocytoma from the thigh. Three primary liver tumours were also collected but matched normal tissue was not available; instead, 3 pieces of normal liver from patients without tumours were obtained (kidney transplant donors). All tissues were placed on ice in RPMI 1640 media for transport to the laboratory and frozen within 30 min in liquid nitrogen and stored at -70 °C until required. Specimens were allowed to thaw at 20 °C and 20% homogenates (w/v) were prepared in ice-cold 0.25 M sucrose. The homogenates were then centrifuged for 15 min at 10 000 x g, followed by 60 min at 100 000 x g. Final supernatants were collected and are referred to as cytosols.

Although tissue obtained from a site adjacent to tumour tissue may not represent true 'normal' tissue, due to the effects of growth factors or hormones possibly secreted by the tumour mass, in this study the term normal represents non-tumour tissue.

6.02 : Glutathione S-transferase activity

Measurement of GST enzymic activity was determined using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The assay was carried out at 37 °C, in duplicate, using 1 mM CDNB and 2 Mm reduced glutathione on a Cobas Fara centrifugal analyzer (Roche Diagnostics Ltd., Basle, Switzerland) (Hayes and Clarkson, 1982) as described in Section 2.

6.03 : Glutathione peroxidase assays

Measurement of selenium-dependent (sGPX) and total glutathione peroxidase (tGPX) utilised an adaptation of the Paglia and Valentine (1967) method for use on a Cobas Fara centrifugal analyzer, as described in Section 2. Hydrogen peroxide (H_2O_2) was the substrate employed to measure sGPX whereas, cumene hydroperoxide (CuOOH) was the substrate employed to measure tGPX.